TOWARD LOCALIZATION CONTROLLABLE PROTEINS: SUBCELLULAR TARGETING TO THE NUCLEUS, CYTOSKELETON, AND PROTEASOME

by

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STATEMENT OF DISSERTATION APPROVAL

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ABSTRACT

The control of subcellular protein localization to alter function offers great potential for treatment of diseases caused by mislocalization. Further, inducing mislocalization may assist in the determination of a protein's function. To this end, three localization-controllable constructs have been created. A central feature among them is their ability to respond only to an externally administered ligand, which is mediated through the incorporation of a steroid hormone ligand-binding domain.

Controlled cytoplasm-to-nucleus translocation can be accomplished through the "protein switch," which was optimized in this work to produce a significant shift in localization from the cytoplasm to the nucleus upon ligand induction. The glucocorticoid receptor ligand-binding domain confers ligand-responsiveness, and nuclear export and nuclear localization signals help shift the balance of localization.

Controlled targeting of proteins to the cytoskeleton can be accomplished through the isolation of the estrogen receptor ligand-binding domain. When this domain binds the antagonist fulvestrant, it forms insoluble protein aggregates that associate with the cytoskeleton. A protein fused to the ligand-binding domain will get sequestered in the cytoskeleton, and any cellular activity it had is lost.

Controlled targeting of a protein to the ubiquitin-proteasome pathway can be achieved by regulating the access of the tumor suppressor p53 to the nucleus, where it binds the protein responsible for its ubiquitination and proteasomal degradation. When p53 was fused to the protein switch, it remained cytoplasmic before ligand

addition, but after ligand it translocated to the nucleus and subsequently sent to the proteasome. This technology has the potential to target other intracellular proteins to the proteasome if a binding domain is incorporated.

This dissertation focuses on the creation and characterization of these localization-controllable constructs so that they may be used in future applications of protein localization control.

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LIST OF ABBREVIATIONS

AR	Androgen receptor
Abl	Abelson Leukemia oncogene
AF	Activation function
BARD1	Breast cancer associated protein
Bcr	Breakpoint cluster region
BRCA1	Breast and ovarian cancer susceptibility protein
CC	Coiled-coil that binds Bcr-Abl
CCmut3	Mutated coiled-coil
CK	Cytokeratin
CML	Chronic myeloid leukemia
CPC	Chromosome passenger complex
CR	Complete response
CRM1	Chromosome region maintenance
CRT	Calreticulin
D3	Dopamine 3 receptor
DBD	DNA binding domain
Dex	Dexamethasone
DNA	deoxyribonucleic acid
FDA	Food and Drug Administration
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin-protein ligase
EcR	Ecdysone receptor
EGFP	Enhanced green fluorescent protein
ER	Estrogen receptor
ER(mut)	ER S167A mutation
ERLBD	Estrogen receptor ligand-binding domain
GR	Glucocorticoid receptor
HECT	Homologous to E6AP carboxy terminus
HER2	Human epidermal growth factor receptor-2
HSP	Heat shock protein
IAP	Inhibitor of apoptosis
ICI	ICI 182,780; fulvestrant
LBD	Ligand binding domain
LMB	Leptomycin B
MAPKK	Mitogen activated protein kinase kinase
MBD	MDM2 binding domain
MDa	mega Dalton
MFP	Mifepristone
MR	Mineralocorticoid receptor

MUR	Muristerone A
Myc	Myelocytomatosis oncogene
NE	Nuclear envelope
NES	Nuclear export signal
NLS	Nuclear localization signal
NPC	Nuclear Pore Complex
NTA	Nuclear translocation assay
OHT	Tamoxifen
PNI	Percentage nuclear intensity
PR	Progesterone receptor
PRR	Partial response rate
PS	Protein Switch
Ran	Ras-related nuclear protein
RanBP1	Ran binding protein
RanGAP1	Ran GTPase activating protein
RanGEF	Ran guanine nucleotide exchange factor
RING	Really interesting new gene
RNA	Ribonucleic acid
RNI	Relative nuclear intensity
SBD	Survivin binding domain
SMAC	Second mitochondrial-derived activator of apoptosis
SV40	Simian virus 40
TD	Tetramerization domain
UBC	Ubiquitin carrier protein, also E2
UID	Ubiquitin interacting domain
XIAP	X-linked inhibitor of apoptosis

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CHAPTER 1

BACKGROUND AND SIGNIFICANCE

Summary

The classic paradigm for treatment of disease has focused on small molecules, peptides, and antibodies that hit "druggable targets" and that are easy to formulate and administer. However, intracellular disease targets may lack small molecules that modulate their function, and protein internalization may not efficiently occur to be an effective option. With heightened technology comes the ability to move beyond that paradigm to more complex means of disease treatment: targeted gene therapy involving delivery through viral or polymer vectors. If limited by lack of small molecules or a druggable target, why not *introduce* a gene that encodes a protein with novel activity, or that replaces the activity of a dysfunctional one? With this technology, one no longer has to find a "druggable target," search a large library of small molecules, or deliver large amounts of a drug in order to reach a therapeutic concentration. A relatively small amount of a therapeutic can be administered, and it will seek out the programmed site. The advantage of polymer and viral delivery is the internalization of the payload—DNA for example. DNA can be engineered in the lab to contain the sequence that encodes a protein with therapeutic function, which can subsequently be introduced into diseased tissue. These tissues will then produce this therapeutic protein on their own. The potential of this technology is enormous,

but it is currently limited by concerns of toxicity and off-target effects. This has not prevented the pharmaceutical industry from creating hundreds of gene therapy drugs with a variety of targets, including cancer, diabetes, and heart disease. Adding a gene that complements what is lacking internally may rescue cells that are experiencing the negative effects, but may have little effect on tissues where that pathway is intact or irrelevant.

To effectively capitalize on the therapeutic potential of gene therapy, there needs to be an arsenal of tools and mechanisms for diagnosis, research, and treatment. The work presented here adds to that arsenal by providing several means of intracellular protein localization control. The subcellular transport and localization of proteins is necessary for cells to function properly—protein may be functional in one compartment, but inactive in another. It is also possible that a protein is *dysfunctional* if it has localized to the wrong compartment, and this dysfunction may lead to disease (1). Correcting this dysfunction is one potential treatment modality, but harnessing the altered function of a protein in a particular compartment is another. Three uses of protein localization control are described in this dissertation, and are illustrated in Figure 1.1.

The first approach is the control of cytoplasm-to-nucleus translocation. This concept has been pioneered in the Lim laboratory, and utilizes a combination of signal sequences in conjunction with a ligand-responsive domain, and it has been named the "protein switch" (2-4). In the unliganded state, the protein switch localizes mostly in the cytoplasm, but after ligand addition it translocates to the nucleus. The optimization of the various domains needed to make the protein switch function will be thoroughly described.

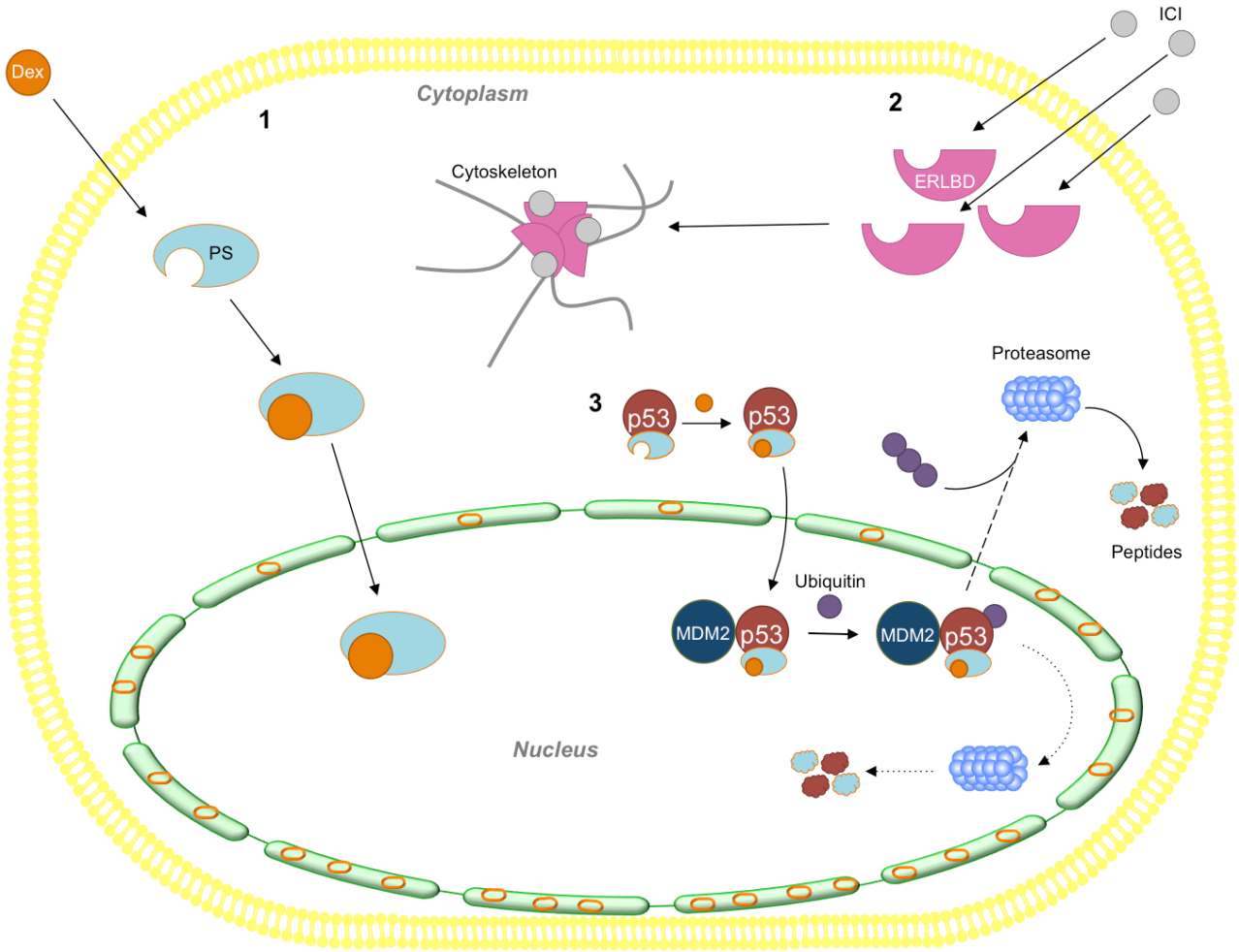

Figure 1.1. Targeting the nucleus, cytoskeleton, and proteasome pathway. 1) shows the binding of externally applied dexamethasone steroid that causes the protein switch to translocate from the cytoplasm to the nucleus. 2) shows that ERLBD will bind fulvestrant (ICI) and aggregate at the cytoskeleton. 3) shows that dexamethasone will cause the protein switch-p53 fusion to translocate to the nucleus where it interacts with MDM2 and is subsequently targeted to the ubiquitin-proteasome pathway.

The second approach is sequestering a protein to the insoluble cytoskeletal fraction. This is accomplished by fusing a protein of interest to the estrogen receptor ligand-binding domain (ERLBD), then adding the drug fulvestrant. Fulvestrant is a member of the selective estrogen receptor downregulator family of drugs, and results in aggregation and accumulation of the receptor in the cytoplasm, followed by proteasomal degradation. However, isolating only the ligand binding domain results in a protein that aggregates at the cytoskeleton in response to fulvestrant, and its cytoplasm or nucleus localization can pre-determined. A protein fused to the ERLBD thus results in aggregation at the cytoskeleton, which blocks the activity of that protein. The design and activity of this cytoskeleton-directed protein will be discussed.

The third and final approach of protein localization control is the targeting of proteins to the degradation pathway. These proteins capable of controlled degradation have been termed "protargs," or proteasomal-targeted protein. Intracellular proteins can be marked for degradation by conjugation with ubiquitin moieties. Ubiquitinated proteins are recognized by the 26S proteasome, which is the major proteolytic machine within the cell. Specific ubiquitin ligases, called E3 ligases, are necessary to add ubiquitin moieties when a protein needs to be degraded. When an E3 ligase is localized in one subcellular compartment, it is possible to control its access to its target protein. An example explored in this dissertation is the interaction of p53 with MDM2—the E3 ligase responsible for the ubiquitination and degradation of p53. When MDM2 is in the nucleus, the entry of p53 to the nucleus can be controlled with the protein switch, and thus the ubiquitination and degradation of p53 can be controlled. By extension, if a protein is fused to this p53, its degradation can be controlled as well. We have also proposed another protarg

based on the response of the estrogen receptor (ER) to the antagonist fulvestrant. This interaction results in proteasomal degradation. While we have proposed this as a viable mechanism for proteasomal targeting, its utility is not yet proven. The initial work will be discussed, but it has not yet demonstrated protein localization control.

Background

Targeting the Nucleus: Cytoplasm-to-Nucleus Protein Switch

The nucleocytoplasmic translocation of proteins can be harnessed to allow external control of an exogenous protein to utilize intracellular transport mechanisms (2, 3). This designed protein has been termed "protein switch" or PS, and is comprised of a nuclear export signal (NES), a nuclear localization signal (NLS), and a ligand-binding domain (LBD). Enhanced green fluorescent protein (EGFP) has been fused to the PS to allow convenient microscopic tracking. After translation, the PS takes on a conformation such that the NES is dominant, and the NLS is at least partially masked, resulting in a predominant cytoplasmic localization. After ligand induction, the conformation of the PS changes, the NLS becomes unmasked, and the protein translocates into the nucleus (3). The process for nuclear import and export that makes this PS possible is briefly summarized here, and more thoroughly discussed in Chapter 2.

Nucleocytoplasmic Transport

Eukaryotic cells have developed effective and selective transport machinery to ensure proteins are in the correct subcellular compartment. While transcription of

DNA happens in the nucleus, mRNA is exported to the cytoplasm for translation. For those proteins needed in the nucleus, such as steroid hormones, histones, and transcription factors, shuttling back into the nucleus must occur (5-10), but this must be done selectively (5, 11). Selectivity is possible because of the nuclear pore complex (NPC): the approximately 125 MDa structure forms aqueous channels in the lipid bilayer of the nuclear envelope at regular intervals (12, 13). These channels form the only site where ions, small molecules, and macromolecules pass. The overall structure is such that an interaction with the karyopharin family of transport receptors is permitted, but nonshuttling proteins are deflected (14-18). The NPC produces a gated, iris-like movement to select for proteins to translocate, but only for macromolecules greater than 39 nm in diameter (40-60 MDa) (19, 20). Smaller molecules may passively diffuse through the pore complex.

Proteins that contain a "classic" NLS are imported into the nucleus by an importin α/β heterodimer (21). Protein import begins in the cytoplasm, with importin α recognizing an NLS. Cargo-bound importin α binds importin β, and translocation through the nuclear pore occurs (5, 22). On the nucleoplasmic side, RanGTP is needed to release the cargo, where it can subsequently function. The import proteins are recycled back to the cytoplasm for more import events to occur (5, 20, 23, 24).

Classic NLSs fall into two categories: those that contain a single stretch (monopartite) of basic amino acids, or those that contain a bipartite sequence with two short sequences of basic amino acids separated by a spacer (12, 25, 26). An example of a classic monopartite NLS is the NLS in SV40 large T antigen (PKKKRKV), which is similar to the one used in the protein switch.

For nuclear export, cargo proteins bind to transporters like CRM1 or calreticulin (CRT) in the presence of RanGTP. This cargo/exporter/RanGTP complex

translocates through the NPC to the cytoplasm, where it is released by hydrolysis of RanGTP (5, 20, 23, 24). The export signals recognize the NES in the protein, which has been classified as a leucine rich sequence (5, 27). These classical NESs are about 10 amino acids in length with hydrophobic residues such as leucine (28-31).

Ligand-Inducibility: Using Steroid Hormone Receptors

Steroid hormone receptors (SHR) are part of the nuclear receptor superfamily of intracellular proteins that regulate gene transcription in response to ligands, and represent "the largest known family of transcription factors in eukaryotes" (32). SHRs include the glucocorticoid receptor (GR), estrogen receptor (ER), progesterone receptor (PR), androgen receptor (AR), mineralocorticoid receptor (MR), and ecdysone receptor (EcR) (33). The genes they regulate are important for growth and development, homeostasis, reproduction, and apoptosis. The general mechanism of SHRs are to bind ligand, release chaperone proteins like heat shock proteins (HSPs), translocate to the nucleus and form homodimers, bind DNA at the hormone response element with the help of coactivators, and initiate transcription (34, 35). The ER binds to the response element sequence AGGTCA, whereas the other SHRs recognize AGAACA (35). Interestingly, as the receptors are binding DNA as dimers, one receptor binds the conserved response element, and the other binds a less conserved sequence exactly three nucleotides away (35).

The structure of steroid hormone receptors is conserved, with a variable N-terminal transactivation region, a DNA binding domain (DBD), a hinge region, the ligand-binding domain (LBD), and a C-terminal domain that also has transactivation functions (32, 35-37). There are two activation function (AF) regions, where AF-1 in the N-terminus has ligand-independent function, and AF-2 within the

LBD has strict ligand dependence (38). The ability of SHRs to respond to ligands comes from the functionality in the LBD, and is crucial to the ability of the protein switch to respond to ligand.

The LBD functions to bind ligand and induce transcription, but also to mediate dimerization, interact with heat-shock proteins, coactivators and corepressors, and mediate nuclear translocation (38-40). LBDs have three main functional regions: the ligand binding pocket, dimerization region, and cofactor binding groove (40, 41). The accepted model for ligand binding is the "mouse-trap" model, which is that ligand binding creates a conformational change in the LBD that shifts the AF-2 region to allow for cofactor binding (42). These LBDs have 12 α-helices, called H1-H12. These helices are arranged into a "three-layered, antiparallel helical sandwich" (38). Together, these three helical sheets form an interior ligand-binding pocket (35, 43-47). The size of the binding pocket correlates with the specificity of the receptor. Larger binding pockets allow for varied ligand structures, whereas smaller binding pockets have higher affinity for fewer ligands. The pocket allows for hydrophobic interactions, hydrogen bonding, and steric specificity for ligands, even among closely related steroid structures (35, 43-47). In the unliganded state, helix 12 is positioned in a way that it cannot interact with coactivators, but a conformational change upon ligand binding shifts its position, stabilizing it against the binding pocket and exposes a region that is able to interact with coactivators (45, 46). Antagonist binding blocks this coactivator interaction, either through blocking the necessary conformation of helix 12 to allow coactivator binding, or by causing helix 12 to mimic the coactivator (44, 48).

These LBDs perform a crucial role in subcellular targeting because of their ability to conformationally respond to agonists or antagonists, creating ligand-

inducibility. This domain influences the subcellular localization in both the liganded and unliganded states. The subcellular localization of the GR, for example, is greatly influenced by the LBD itself. Without ligand, the GR is cytoplasmic, but becomes nuclear upon agonist binding. Replacing the LBD of the PR, a predominantly nuclear receptor, with GRLBD changes the localization to predominantly cytoplasmic (49-53). In the cytoplasm, the receptor interacts with the chaperone protein HSP90, which is partially responsible for maintaining the receptor in this compartment (52-59). The HSP90 inhibitor geldanamycin blocks the interaction with GR, and results in nuclear translocation of the receptor. This translocation is possible due to two NLS signals located in the LBD (37, 59-62). Upon ligand binding, HSP90 is released, and localization signals are exposed, and the receptor translocates to the nucleus through the NPC (53, 63-66).

Protein Switch: Design and Applications

The protein switch is designed to be a means to correct or exploit the function of mislocalized proteins. Mislocalized proteins include tumor suppressors (p53, BARD1, BRCA1, INI1/hSNF5), transcription factors (FOXO, NF-kB), cell signaling proteins (β-catenin, rhodopsin, vasopressin receptor, LDL receptor, CTFR, dopamine D3 receptor), and may be mislocalized from virtually any part of the cell (Chapter 2, Davis et al. 2007 (1) and references therein). The bi-directional protein switch developed in our lab is capable of cytoplasmic-to-nuclear translocation when bound to cognate ligand. This research focuses on the selection of appropriate signal sequences to balance the cytoplasmic and nuclear localization upon induction, and a ligand-binding domain that produces the right conformational change that exposes the nuclear localization signal in order to create the translocation response.

The first protein switch was conceived of, and designed by, Charu Kanwal. It contained a nuclear export signal (NES) and a ligand binding domain (LBD) that is responsive to an external ligand (4). EGFP was selected as a gene of interest to track the localization of a protein construct using fluorescence microscopy (4, 31, 67-70). In the absence of external ligand ("off" position), the protein construct was mostly present in the cytoplasm due to the dominant effect of the NES. Upon ligand addition ("on" position), the protein construct translocated to the nucleus. This process is thought to occur due to a change in conformation of the LBD that masks the NES and harnesses the inherent translocation properties of steroid hormone receptors. This response was found to be dose-dependent (4). One conclusion drawn from this work was that the relative strength of the NES had an impact on the initial localization of the construct; a strong NES may overpower the ability of the protein to translocate to the nucleus. To build on this data, the optimization of this protein switch was then to make the "off" state more cytoplasmic and the "on" state more nuclear. The utility of the protein switch constructs lies in the ability to keep the attached therapeutic protein in one cellular compartment when inactive, and upon addition of ligand, controlled movement to another compartment where it has activity (or blocks activity, as the case may be).

The first protein switch used a truncated form of the LBD from the progesterone receptor that is responsive to mifepristone (RU486) (71), and non-responsive to natural agonist progesterone or synthetic agonist R5020. The benefit of an LBD with this ligand response is that naturally occurring hormone in the body would not trigger the translocation of the switch, meaning that it can be only externally controlled. A drawback of this ligand choice is that mifepristone can antagonize wild-type endogenous progesterone and glucocorticoid receptors, and is

also used as an abortifacient (4). The need for this ligand is sub-optimal if this system is to ever reach the clinic, so a new LBD that responds to a ligand with fewer side effects or controversies was warranted.

As previously mentioned, further optimization included making the protein switch more cytoplasmic in the unliganded state, and more nuclear in the liganded state. We hypothesized that adding a nuclear localization signal (NLS) to the protein switch may increase the nuclear translocation effect. The optimization revolved around balancing the relative NES and NLS strengths on the localization of constructs. The work thoroughly discussed in Chapter 2 focuses on the alternative NES, NLS, and LBD combinations that were examined in the optimization of the protein switch.

Targeting the Cytoskeleton: Induced Interaction by Estrogen Receptor Ligand-Binding Domain

One of the challenges of the protein switch optimization was that an LBD needed to produce a conformational change that tipped the balance of signal sequences in order to cause translocation. One such LBD that we tested was from the ER. During these experiments we learned that the ER antagonist fulvestrant causes cytoplasmic accumulation of the full-length receptor, with a transient association with cytokeratins 8 and 18 (CK 8/18) before finally being degraded by the proteasome. However, isolating the LBD portion alone did not result in proteasomal degradation, but did result in aggregation of our construct. Through a series of experiments delineated in Chapter 4, we found that fulvestrant caused the LBD to aggregate at the cytoskeleton.

The cytoskeleton of a eukaryotic cell is a dynamic structure comprised of proteinaceous filaments that fall into three types: intermediate filaments, microtubules, and actin filaments (also called microfilaments) (72-74). The intermediate filaments impart mechanical strength and organize the three-dimensional structure of the cell (73, 75), the microtubules direct intracellular transport, and actin filaments influence the shape and protuberances of the cell, and participate in cell interactions with the environment (76). These proteins are able to rapidly rearrange and adapt to cellular needs and environmental conditions. For example, microtubules are most commonly associated with mitotic spindle formation, but also arrange as cilia and flagella that create cell or extracellular movement. Further, they can also be arranged as the transport network for materials within the cell, such as DNA and protein. Actin creates the contractile ring for cell division, but can also serve as the structure that creates muscle contraction. Intermediate filaments function intracellularly to encage the nucleus, and extracellularly to create strong structures, like hair and nails (74).

Intermediate filaments include cytokeratins (keratins; CK), vimentins, and lamins, and all types are not necessary in every cell type. Cytokeratins, expressed in all types of epithelial cells, are the most diverse group in the intermediate filament category, with about 20 types in human epithelial cells, and at least 10 more are specific for hair and nails (74). The CKs can be divided into acidic (type I; CK9-19) or basic (type II; CK1-8) (72, 73, 77). Cytokeratin filaments come together in equimolar amounts to form heterodimers of type I and type II CKs, and the resulting chains dimerize to form a tetrameric subunit (72-74, 77). Multiple types of CKs within a cell will copolymerize to form a single network. Their diversity is useful in cancer diagnosis because a specific subset of CKs found in the tumor may hint at the

epithelial tissue from which the tumor originated (74). Indeed, CKs have been useful as immunohistochemical markers for diagnosis of tumor pathology (78).

Cytokeratins in Cancer

Adenocarcinomas arise from glandular tissue, and collectively comprise the majority of epithelial malignancies throughout the body (73, 78). Most adenocarcinomas express CK8 (and its obligate partner CK18), CK19, and variably CK7 and CK20 (72, 78). Both ductal and lobular types of breast adenocarcinomas constitutively express CK7,8/18, and 19 (72, 79). The CK7 positive and CK20 negative phenotype has been associated with ovarian, endometrial, lung, salivary gland, and thyroid adenocarcinomas, as well as most mesotheliomas (72, 78, 80-82). Gastrointestinal cancers like pancreatic, esophageal, gastric and biliary tract adenocarcinomas are all CK7 positive, with variable CK20 positivity (80). Cytokeratin 7/20 negative cancers include prostate and hepatocellular carcinomas (72, 83).

In addition to diagnostic value, CKs also have value as prognostic markers in epithelial malignancies. For example, reduced expression of CK8 and CK20 is associated with tumor aggressiveness and decreased survival in patients with colorectal cancer (72, 84). There is also a well-defined relationship between cytokeratin expression and breast cancer. The basal-like subtype is defined by CK5/6 positivity, and ER, PR, and human epidermal growth factor receptor-2 (HER2) negativity, and is associated with poor prognosis and low disease-free and overall survival (72, 85, 86). Downregulation of CK18 is associated with advanced tumor grade, bone metastasis, and decreased survival in metastatic breast cancer (72, 87-90), and 25 to 80% of all breast carcinomas have loss of CK18 expression (90-93).

Interestingly, while loss of CK expression is correlated with poorer prognosis in some tumor types, CK expression can protect against cellular stressors such as death receptor activation and chemotherapy. For instance, mice with CK8/18 knockout are more susceptible to apoptosis mediated by Fas (extrinsic apoptotic pathway). Over-expression of CKs by transfection can impart multidrug resistance to chemotherapeutic agents (72, 94-97).

The Estrogen-Receptor Aggregates to the Cytoskeleton

The estrogen receptor (ER) is a member of the steroid hormone nuclear receptor (SHR) family (98), and as such it has an N-terminal activation domain, a central DNA binding domain, and a C-terminal ligand binding domain (LBD) (32), and functions as a transcription factor when bound to ligand (36, 44). The stability of the ER has been shown to be dependent upon the ligand to which it is bound (99). Estradiol destabilizes ER, tamoxifen stabilizes, and fulvestrant (ICI) results in cytoplasmic aggregation and subsequent proteasomal degradation. Fulvestrant, a clinically useful antagonist of ER, is a member of the selective estrogen receptor down-regulators (SERDs) that act to disrupt the typical nucleocytoplasmic shuttling of ERα and causes rapid degradation (100, 101). The bulky side chain of fulvestrant induces a conformational change of helix 12 within ERα's LBD that increases hydrophobicity and results in receptor insolubility (102, 103). After insolubility comes proteasomal degradation, likely due to cytokeratins 8 and 18, which facilitate proteasomal degradation of ERα (104). However, proteasomal degradation requires a functional helix 12, and an active transcriptional activation complex (105-107). Cytokeratins 8 and 18 are the primary intermediate filaments of single-layer epithelial cells (78). We hypothesized that a GFP fusion protein with the ligand-

binding domain of ER (ERLBD) will target the insoluble cytoskeletal fraction when bound to fulvestrant. Isolating the LBD from ER will still provide a LBD for ligand control, but will not result in transactivation *or* proteasomal degradation because it lacks the other domains necessary for those processes. A construct capable of forming insoluble aggregates could be useful in sequestering active proteins, rendering them inactive.

Targeting the Ubiquitin-Proteasome Pathway: p53 and ER Protargs

In the previous section, it was mentioned that we learned of the response of full-length ER to fulvestrant binding: receptor degradation by the proteasome. While we did examine what would happen if only the LBD was isolated, the potential to *target* the proteasome did not escape our attention. This led us to the hypothesis that the ubiquitin-proteasome pathway could be targeted if a fusion protein was constructed that contained a region that would ultimately get ubiquitinated and sent to the proteasome for degradation. We termed these constructs "protargs." Several such protargs were proposed, and two are discussed in this dissertation. The first is based on the p53/MDM2 interaction, and the other was utilizing full-length ER.

Sending proteins to the ubiquitin-proteasome pathway is another means of controlling a protein's function by localization. Intracellular proteins that are associated with disease, such as Bcr-Abl in chronic myeloid leukemia (CML) (108-110), inhibitors of apoptosis such as survivin and XIAP (111, 112) and MDM2 (113, 114) present themselves as interesting targets for therapy. Many of these disease-associated proteins have small molecule inhibitors that are effective (tyrosine kinase

inhibitors against Bcr-Abl, for example (115)) but others do not have druggable domains. Survivin is a member of the inhibitor of apoptosis (IAP) family, whose over-expression has been associated with a wide array of cancers, and thus far small molecules have not successfully been developed against survivin, so novel gene therapy approaches may be particularly instrumental in blocking its activity (116-118). We hypothesize that creating a protein capable of exploiting the ubiquitin-proteasome system will direct these proteins to the proteasome, and may be an effective therapeutic modality. The first protarg described is based on the tumor suppressor p53 and its induced proteasomal degradation by MDM2. This work has led to a manuscript submitted for publication, but the ER protarg has yet to be proven as an effective proteasome targeted protein. The foundation of this concept is introduced here.

The ubiquitin-proteasome system is the major proteolytic system in eukaryotes, "with critical function in cell cycle control, apoptosis, inflammation, transcription, signal transduction, protein quality control, and many other biological processes" (119), and is integral to the balance of cellular anabolic and catabolic pathways (120). It is estimated that 80 to 90% of cellular proteins are degraded by the proteasome in both the cytoplasmic and nuclear compartments (71, 121-124). The involvement of the proteasome in the degradation of oncogenes and tumor suppressors has drawn attention to the proteasome as a potential tool in cancer therapy (125, 126). The process begins with an enzyme cascade involving E1, E2, and E3 enzymes that together coordinate the conjugation of ubiquitin to a target protein. The processes of proteasome recognition and degradation of proteins will be discussed here.

The 26S Proteasome

The proteasome is a large protease, with a size of over 2.5 mDa. It functions as a protease that degrades proteins conjugated with ubiquitin moieties (119, 123, 127). The proteasome is responsible for recognition of proteins marked for degradation, protein unfolding, and ubiquitin hydrolysis—all distally from the actual site of proteolysis. These mechanisms are mediated by the different subunits of the proteasome. In order to protect the cell from unregulated proteolytic activity, the proteasome has a barrel shape, which the proteolytic activity occurring internally, restricting access to only those proteins recognized and unfolded by the "lid" structure (128, 129).

The proteasome can be split up into two structurally and functionally distinct units: the 19S regulatory lid, and the 20S core particle (120). The 20S proteasome contains 28 subunits, with two copies each of seven distinct α and β subunits. The barrel shape comes from two rings of β-type subunits capped on top and bottom with a ring of α-type subunits, such that the arrangement is [(α1- α7)(β1- β7) (β1- β7) (α1- α7)], with pseudo seven-fold symmetry (120, 130). This subunit arrangement yields three inner compartments, two antechambers, and a proteolytic chamber. The proteolytic chamber is formed by the β-type subunits (120). The 19S regulatory unit is V shaped, and caps the 20S core on both ends (131, 132). The 19S particle, often referred to as the regulatory particle, is composed of 19 subunits, six ATPases that form the base, and 13 regulatory subunits (133). The base is proximal to the core, and is comprised of six ATPases (Rpt1-6) of the AAA family (134, 135). These ATPases form a pseudo-symmetrical ring structure that forms the interface with the α-type subunits of the 20S core (119, 129, 136-138). The ATPases function to catalyze the assembly of the 19S and 20S complex into the 26S proteasome, gating the entry

of proteins into the catalytic core, and to bind, unfold, and translocate proteins into the core (128, 131, 135, 139-142). The 13 regulatory subunits are termed Rpn1-3, Rpn5-13, and Rpn15. The lid consists of Rpn3/5/6/7/8/9 (119, 143). The Rpns are positioned in such a way that Rpn8 and 11 are at the tip, which are the subunits involved in deubiquitination (143).

Substrate Recognition and Deubiquitination

Proteins tagged with at least four ubiquitin moieties are recruited to the proteasome for degradation (144, 145). Rpn10 and 13 are the subunits of the 19S proteasome responsible for recruiting ubiquitinated substrates (143, 146). A distance of 90 Å separates these two subunits, which is the length of 4 ubiquitin moieties (144, 145). A commitment step follows initial docking of the protein (147). Lasker et al. have recently put forth a hypothesis that other subunits in the regulatory particle, namely Rpt1/2 and Rpt4/5, rotate to swing Rpn10 and Rpn13 closer to Rpn11 to allow access to the ubiquitinated protein (143). This motion may also open the gate to the core. Rpn11 recognizes ubiquitin chains at the proximal ubiquitin to the protein to which it is attached, resulting in whole ubiquitin chain cleavage, a process that is ATP-dependent (148). As a side note, there are other deubiquitinating enzymes can remove ubiquitins before they reach the proteasome (Ubp6 and Uch37), but they antagonize protein degradation rather than facilitate it (149).

The proteolytic activity of the proteasome originates from the β-type subunits, which are part of the N-terminal nucleophile hydrolases superfamily (150, 151). The amino acids necessary for proteolytic activity are N-terminal thr-1, glu-17, lys-33, and asp-166. The N-terminal thr-1 on each β-type subunit is a nucleophile that attacks the carbonyl carbon of a peptide bond with its side-chain amino group and a

water molecule (152-156). The main proton acceptor is the main-chain amino group (157). The proteolytic activity of the proteasome is often referred to as having caspase-like, trypsin-like, and chymotrypsin-like activity (158). This is due to the preferences that different β-type subunits have for amino acid residues. β1 prefers acidic residues, β2 cleaves on the carboxyl side of lysine or arginine, and the β5 cleaves after hydrophobic residues, but these enzymes have relatively low specificity (157). As the protein is degraded, peptides are trapped within the chamber until they are about seven to nine residues in length (159). This is likely related to the physical distance between active sites within the proteasome (119).

Ubiquitin and its Ligases

Ubiquitin is a protein with 76 amino acids. As previously mentioned, ubiquitin is conjugated to proteins in a coordinated effort by ubiquitin enzymes. This may occur singly, called monoubiquitination, which may not result in protein degradation. Monoubiquitination has functions like histone processing, transcriptional activation, endocytosis, DNA repair, and endosomal sorting (160-164). Polyubiquitination is the global signal for proteasomal degradation.

The first step in protein ubiquitination is the creation of a thioester linkage between ubiquitin and a ubiquitin-activating enzyme (E1) in an ATP-dependent manner. The next step is trans-thiol esterification of the ubiquitin to a ubiquitin-conjugating enzyme (E2; ubiquitin carrier protein (UBC)). A ubiquitin-protein ligase (E3) catalyzes the transfer of ubiquitin to the ε-amino group of a lysine residue within the target protein (165). Lysine residue 48 becomes a possible site for ubiquitination, which is necessary for chain formation (122, 163, 166). Chain elongation may involve other factors aside from E2 and E3. The specificity of E3

ligases is such that they recognize few proteins, which means there are many E3 ligases (~600 in mammals) within the cell (167, 168). Specific combinations of E2 and E3 enzymes must come together, but there are fewer E2s in the cell (~40 in mammals) (163, 167). E1, E2, and E3 enzymes do have some functions unrelated to ubiquitination, such as SUMOylation and NEDDylation, which are other post-translational modifications for intracellular signaling (169).

E3 ligases belong to one of three families: the HECT (homologous to E6AP carboxy terminus), the RING (really interesting new gene) finger, and U-box domain types (163, 167, 170). These different E3 families have separate mechanisms for transferring ubiquitin to their target protein.

HECT domain E3s have roles that regulate cellular growth, protein trafficking, and the immune response (167, 170). The HECT domain is comprised of about 350 amino acids in the C-terminal end of the protein. The N-terminal domains are varied to allow for diverse substrate targeting. Within the C-terminal HECT domain, there are two lobes, where one interacts with the E2, and the other contains the active site that forms a cysteine thioester intermediate with ubiquitin (171). It is proposed that these two lobes are connected by a hinge that allows them to swing together for ubiquitin transfer with E2 enzymes (167, 171-173).

While HECT E3s form a thioester intermediate with ubiquitin, the RING finger E3s provide direct transfer. RING finger domains have a Zn^{2+}-coordinating domain with cysteine and histidine residues that mediate E2-dependent ubiquitination (174). Two Zn^{2+} ions and amino acid residues form a scaffold that brings the E2 and target protein together (175). An interesting property of the RING finger members is that they can act as monomers, acting independently to ubiquitinate proteins, or they may act with a dimer partner, forming homo- or

heterodimers. Examples of E3s requiring homodimerization are inhibitors of apoptosis, RNF4, SIAH, and TRAF2. Examples of E3s with heterodimer formation are MDM2 with MDMX, and BRCA1 and BARD1. The requirement for some E3s to form heterodimers is that one RING domain alone lacks the ability to stabilize the E2-binding RING domain (167).

U-box domain E3s are similar to RING domain ligases, but do not have Zn^{2+} coordinating the ubiquitin transfer, but do so with hydrogen bonds and salt bridges. An example of a U-box ligase is CHIP, a ligase that acts as a homodimer and requires protein chaperones, like HSP90, HSP70, and HSC70 (176-178). CHIP participates in sending proteins to the degradation pathway, but is also involved in chaperone-assisted protein folding (167, 178, 179).

The Proteasome Pathway in Cancer Therapy

Clearly the proteasome and the proteins it degrades have major consequences in a broad range of cellular functions—both positive and negative. The impact that the proteasome has on proteins involved in cancer has drawn attention to the proteasome and the ancillary proteins in the pathway as druggable targets. The typical approaches to target this pathway have focused on inhibiting the proteasome directly by blocking the catalytic activity of the β-type subunits, or by inhibiting the E3 ligases that affect a protein of interest. The most famous example of a proteasomal inhibitor is bortezomib (Velcade®), the first Food and Drug Administration (FDA)-approved drug in its class, and indicated in the treatment of patients with multiple myeloma (180). The FDA has recently approved a new proteasome inhibitor, carfilzomib (Kyprolis®), indicated for patients with multiple myeloma who have progressed after prior therapies, including bortezomib (181).

MDM2, the E3 ligase for p53 has been targeted, with at least two drugs in clinical trials (RO5045337, JNJ-26854165) (182). Rather than inhibiting the proteasome, we sought to utilize it—we wanted to send proteins deliberately to the proteasome for degradation. The first protarg discussed here is based on p53 and MDM2. The MDM2/p53 interaction that causes proteasomal targeting is discussed later in this introduction, and in Chapter 5.

p53 Tumor Suppressor

p53 was the first tumor suppressor to be identified, and now it is the most widely recognized and well-characterized tumor suppressor (183). First described in the literature in 1979, the name p53 came from its size: 53 kDa. It was initially thought to function as an oncogene because of its high expression in transformed cell types, and relatively low expression in nontransformed types (184-187). Further, it was thought that mutations in p53 enhanced its transformation efficiency (188). Ultimately, it was discovered that the original cDNA used in experiments was a mutated inactive form, and subsequent studies demonstrated conclusively that p53 is indeed a tumor suppressor, not an oncogene (189).

We now know the main functions of p53: to act as a transcription factor, cell cycle regulator, and initiator of apoptosis. More specifically, it binds to regulatory regions in the DNA of specific genes involved in regulating the cell cycle and induction of apoptosis in response to stress. p53 is maintained at low levels, but in response to stress it accumulates as a function of increased p53 translation and decreased p53 degradation (190). The accumulation occurs in the nucleus, which promotes the transcription of genes associated with apoptosis and cell cycle arrest. This can occur due to DNA damage, hypoxia or hyperoxia, nucleotide depletion,

aberrant growth signals, chemotherapeutic agents, ultraviolet light, or oncogene activation (189).

Inactivating mutations in p53, or loss of p53 altogether, was found in nearly half of human cancer types, and is thought to be the most commonly altered gene in human tumors (189, 191, 192). In cancers where the gene is not inactivated, post-translational modifications and other protein interactions may inactivate the function of p53 (193-195). As p53 is a nodal protein, interconnected to many proteins that perform important roles in cell cycle and cell death, it is unsurprising that loss of p53 function is selected in most cancer types (196).

p53 Structure and Tetramerization

The p53 protein specifically binds to target sequences as a tetramer (197). Wild-type p53 (α-isoform) consists of 393 amino acids (aa), with five functional domains. These domains are: two N-terminal transactivation domains (TD1: aa 1–42, TD2: aa 43–63), a proline-rich domain (PRD: aa 64–92), the central DNA-binding domain (DBD: aa 102–292), the C-terminal tetramerization domain (aa 326–356) and basic domain (aa 364–393) (189). It is important to note that at least 10 different isoforms of p53 have been described in the literature, and are beyond the scope of this dissertation (198, 199).

The transcriptional activation domain is necessary to recruit transcriptional machinery, such as the TATA box binding protein (TBP) and TBP associated factors (TAF) of the initiation complex (200). The N-terminus contains the location of MDM2 binding and the proline-rich domain (amino acids 63-97) that is necessary for p53-mediated apoptosis and tumor growth suppression (201, 202). The DNA binding domain contains the region that binds to the consensus target sequence, and also

contains the most mutations (189). The tetramerization domain is necessary for transactivation of p53 and tumor suppression, as the active form of p53 is tetrameric (203). p53 forms an antiparallel dimer of dimers, that is two monomers form a dimer, with which it forms another antiparallel dimer (204). The C-terminus contains regulatory regions that regulate DNA binding, transcription activation, and post-translational modification (189).

As previously mentioned, mutations of p53 occur in about half of human tumors, and the majority of these mutations are located in the DBD, concentrated in only a few nucleotides, which influences the binding of p53 to its target sequences (189, 205). Some of these nucleotide changes result in the exchange of amino acids that make contact with the DNA, which eliminates or weakens the binding of p53 to DNA, or even alters the sequence specificity of the mutant p53. Mutant p53 may actually become oncogenic (206).

Regulation of p53

The amount, localization, and activity of p53 can be posttranslationally regulated with modifications like phosphorylation, acetylation, ubiquitination and attachment of ubiquitin-like moieties such as SUMO and Nedd8 (207). The primary phosphorylation sites are at the N-terminal Ser and Thr residues. As these reside in the MDM2 binding domain, phosphorylation affects p53 stability (i.e. proteasomal degradation), and p53-associated apoptosis or senescence. Acetylation on the C-terminus may block ubiquitination on critical lysine residues; these modifications stabilize p53 and promote apoptosis and senescence (208).

MDM2-p53 Interaction

MDM2 is the predominant ubiquitin ligase regulating p53 (209). MDM2, or murine double minute clone 2, is classified as an oncogene, and it is the major down-regulator of p53 (190). It is a member of the RING family of E3 ligases (210) that was originally identified as a protein involved in abnormal cellular proliferation and tumorigenesis (211), and was found to be over-expressed in osteosarcomas and soft tissue tumors (195). The feedback loop of p53 with MDM2 is such that p53 binds to the regulatory region of the MDM2 gene, which stimulates transcription of the gene and ultimately increased MDM2 protein levels. As MDM2 levels rise, p53 modification by ubiquitination increases, and p53 is degraded. As p53 levels decrease, less MDM2 is made (212, 213). Mouse genetic studies have demonstrated that MDM2 can regulate p53 activity in embryonic cells, preventing p53 from inducing apoptosis inappropriately (214).

The signal for proteasomal degradation is attachment of ubiquitin to the C-terminal lysine residues of p53 by MDM2, which is a specific E3 ligase for p53 (207). The interaction of MDM2 with p53 occurs in the N-terminal region, with MDM2 transferring a ubiquitin to p53 and thereby beginning the path to degradation (210). This monoubiquitinated p53 is shuttled out of the nucleus via the classical export pathway mediated by CRM1. p53 also contains an NES that is masked by tetramer formation, and the loss of tetramerization by ubiquitination also results in increased export of the protein (215). The MDM2/p53 interaction also blocks the transactivation function of p53 by binding a region that is necessary for increasing RNA polymerase II-mediated transcription, so genes for cell cycle arrest and apoptosis are not upregulated (216). As levels of MDM2 rise, transactivation of p53

is blocked, and p53 is marked for degradation and levels fall, leading to a commensurate drop in MDM2 levels (190).

The role that MDM2 plays in p53s activity naturally draws focus to how it may be involved in the development of tumors. Tumor types with over-expressed MDM2 may proliferate even with wild-type p53, but this is only noted in 7 to 20% of tumors (most notably soft tissue tumors and sarcomas) (190, 217).

Crystal structure analysis of MDM2/p53 reveals that MDM2 binds p53's transactivation domain, with p53 having amino acids phe19, trp23, and leu26 sliding deeply into a cleft in the N-terminus of MDM2, required for binding to transcriptional machinery (218).

Transfer of the ubiquitin moiety from an E2 ligase depends on the domain of MDM2 (219). While MDM2 is necessary and sufficient for ubiquitination of p53, its homologue MDMX is necessary for proper p53 regulation. MDMX also contains a RING domain, but does not demonstrate ubiquitin ligase activity towards p53 (220). MDMX regulates p53 by forming heteromers with MDM2 and enhances MDM2-mediated ubiquitination of p53. The importance of MDMX is highlighted by knockout studies that demonstrate embryonic lethality (207). Like MDM2, MDMX is frequently over-expressed in tumors with wild-type p53 (206).

The ubiquitination of p53 by MDM2 begins with the binding of MDM2 to the N-terminal BOX-I region of p53. This single interaction is not enough for MDM2 to ubiquitinate p53; small molecule mimetics of this interaction do not prevent the E3 ligase activity of MDM2 (221-225). A second region within p53, called BOX-V, was determined to be capable of providing a ubiquitination signal and docking site for MDM2 (222, 226). BOX-V-like peptides that bind MDM2 are potent inhibitors of the ubiquitination action of MDM2. This led Wallace et al. to propose a model where

binding of MDM2 to the N-terminal BOX-I region of p53 allosterically promotes binding of the acidic domain of MDM2 to the BOX-V region (222, 227). In other words, the allosteric activation of MDM2 by the N-terminal BOX-I region allows a conformation in MDM2 to bind the BOX-V region within p53s DBD to allow E2 transfer of ubiquitin to the C-terminus of p53.

p53-Directed Therapies

Several different approaches to restore or promote p53 action are described in the literature, and range from small molecule inhibitors of p53 regulators, to replacement of the *TP53* gene. p53 therapies may seek to restore wild-type p53 activity by introducing wild-type p53 to replace the lack of tumor suppression by mutant p53 (228-230). Cell-based experiments have shown that the ectopic expression of wild-type p53 induces apoptosis or senescence in cells with mutant p53 (231-236). A drawback of this approach is that in cells with mutant p53, oligomerization between endogenous and exogenous p53 may occur, and the effect of the wild-type protein is dampened or abrogated entirely (237). However, p53-negative tumors may respond highly to this approach (238).

The most commonly used methods to introduce exogenous wild-type p53 are delivery by retroviral and adenoviral vectors (239). While retroviral vectors integrate their DNA into the genome, adenoviral vectors do not (240). Integration of viral DNA may itself result in tumorigenesis. Adenoviral vectors are able to infect non-proliferating cells, but normal cells appear to be largely unaffected by the over-expression of p53 (241). Advexin®, a replication-deficient adenovirus carrying wild-type p53, has been tested in clinical trials, but the FDA has not approved it for use in the United States. In China, a virally delivered form of p53, called Gendicine®, is

already being used therapeutically for head and neck squamous cell carcinoma with some success (242). The complete remission (CR) and partial response (PRR) rates in phase I/II studies were 64% and 29% respectively in combined therapy of Gendicine and radiotherapy. Radiotherapy alone produced a CR of 19% and PR of 60% (243). These results are not without controversy as the clinical trials in China are considered to be less rigorous than in the United States, and some experts consider the trial methodology to be flawed (244).

Small molecule therapeutics have been developed to compensate for specific p53 mutations that prevent necessary protein interactions or result in destabilization of the protein. Three compounds with proven activity are PRIMA-1 (p53 reactivation and induction of massive apoptosis), CP31398, and PhiKan083 (206). PRIMA-1 restores DNA binding of p53 with several different mutations in the DNA binding domain, and results in the activation of the mitochondrial apoptosis pathway via upregulation of Puma and Bax (245, 246). CP31398 compensates for the destabilization of the DNA binding domain due to mutations in p53, possibly by alkylating cysteine residues. The stabilized protein was able to upregulate the cell cycle inhibitor p21 and induced apoptosis (206). PhiKan083 also functions by stabilizing the Y220C mutation commonly seen in p53 (206). Many other small molecules are in development that similarly restore activity of mutant p53, but they will not be discussed here.

MDM2-Directed Therapies

Targeting MDM2 is a means of reactivating p53 in tumor cells with wild-type p53, which means interruption of the cell cycle and/or apoptosis (182). MDM2 is over-expressed in certain tumor types, so it is an attractive target for cancer therapy.

MDM2 over-expression negatively correlates with prognosis in sarcoma, glioma, and acute lymphocytic leukemia (114, 182), and inhibition of MDM2 has shown growth inhibition of lymphoma, sarcoma, and hepatocellular carcinoma in animal studies (182, 247). Over-expression of MDM2 is the result of single nucleotide polymorphisms in the promoter region (248), and the result is loss of p53 activity. A diverse group of compounds has been identified by high-throughput screening, as well as by computational database screening, that inhibit the p53-MDM2 interaction. These small molecules target this interaction by inhibiting MDM2, blocking MDM2 binding on p53, or inhibiting the E3 ligase activity of MDM2 (182). The most promising of these compounds directed against MDM2 are the small molecule inhibitors called nutlins (206). Nutlins are imidazoline compounds that displace p53 by mimicking the BOX-I region, binding the p53 pocket of MDM2 with nanomolar potency (90 to 260 nM) (249, 250). Currently, there are three nutlins, nutlin-1, -2 and -3, which share a common structure but differ in their substituting moieties. These nutlins inhibit MDM2 because they mimic the key amino acids in p53 that MDM2 recognizes. The result is that p53 is stabilized, target gene expression is induced, and cell cycle arrest or apoptosis follows. The pro-apoptotic function of nutlin-3 (RO5045337) has been examined in several tumor cell lines and primary lines (250-252). These experiments demonstrated a requirement for wild-type p53 for nutlins to have a cytotoxic effect. This drug is already in early-phase clinical trials, and is one to watch as an agent with potentially interesting outcomes.

p53 Protarg: Design and Applications

We propose a novel proteasome targeting, or “protarg” system. This will be a protein fusion of a control domain, a ubiquitin-interacting domain (UID), and a

functional domain—one that interacts with the intracellular environment, or one that can dimerize with an endogenous protein. The degradation of protargs will be induced when ubiquitin ligases interact via the UID and subsequently translocate to the proteasome for proteolysis. The protarg tested will be based on the p53-MDM2 interaction, where the access of MDM2 to p53 will be under control of externally applied ligand. The protein switch (PS) previously described and elucidated in Chapter 2, will be applied as the control domain. Fused to the PS will be a p53 domain capable of interacting with—and being ubiquitinated by—MDM2. The functional domain is not explored in this work, but will be discussed in the final chapter. We hypothesized that the PS would dictate the localization of the p53 domain such that without ligand, it would be cytoplasmic, and therefore untouched by MDM2. With PS induction by dexamethasone, the construct would translocate to the nucleus and interact with MDM2, and ultimately result in nuclear export and proteasomal degradation.

ER Protarg

The human estrogen receptor is a member of the nuclear hormone family of transcription factors that, when activated by agonist, binds to an estrogen responsive element (ERE) located within the regulatory sequences of target genes (253). The receptor has three main functions: hormone-independent transactivation through the N-terminal AF1 domain, DNA binding through the DNA binding domain, and ligand binding and transactivation through the ligand-binding domain (LBD) (32, 36, 253, 254). The endogenous hormone that activates estrogen receptor is estradiol, which acts to induce a conformational change of helix 12 (within the LBD) that allows for coactivator binding and subsequent interaction with EREs.

Further, estradiol binding induces ER proteasomal degradation. Much research has been done to determine the effect of ligands on the conformation of helix 12, and receptor degradation. The pure antagonist fulvestrant has been found to block all ER transactivation, and potently induces the proteasomal degradation of the receptor (99, 100, 104, 255, 256). Fulvestrant binding induces a unique conformational change in helix 12 that allows for the interactions with cytokeratins 8 and 18 (CK8/18), and simultaneously recruits ubiquitin ligases to begin the degradation targeting process (104, 107). These CK8/18 intermediate filaments are closely associated with proteasomes, and this is thought to facilitate the quick degradation of the ER. We propose to exploit this efficient degradation process to create a ligand-controllable protarg responsive to the ligand fulvestrant. This system will provide the most control, resulting in on/off proteasomal degradation: on in the presence of fulvestrant, off in its absence. The estrogen receptor used in these studies needs to be capable of degradation upon fulvestrant binding, but it should not transactivate gene expression if bound to endogenous hormone estradiol. Work by Valley et al. showed that the mutation of a serine 167 to alanine abolished a phosphorylation event necessary for complete transactivation of the receptor, but maintained the ligand-induced degradation (105-107). Other groups have attempted to determine critical amino acid residues necessary for fulvestrant-induced degradation, but all have been done in a full-length ER context, with results varying between cell lines (107, 257, 258). Thus, to retain fulvestrant-induced degradation without introducing a receptor capable of promoting cellular proliferation, a full-length receptor with the S167A mutation will be used. The first use of this particular protarg was the intracellular inhibitor of apoptosis (IAP) survivin.

Survivin as a Cancer Target

Survivin is a member of the inhibitor of apoptosis (IAP) family of proteins, undetectable in differentiated tissue, but over-expressed in embryonic and cancerous cells (259). Survivin is one of a few nodal proteins involved in multiple signaling pathways implicated in tumor progression (112). Initially characterized by Altieri and colleagues in 1997 (259), an immense body of research has been conducted on survivin in the last 12 years, including clinical trials of inhibitors of this important cancer pathway. Altieri best summarized survivin's importance as "…one of the most tumor-specific molecules, which antagonizes apoptosis, promotes tumor-associated angiogenesis, and acts as a resistance factor to various anticancer therapies" (112). Many potential roles for survivin in the inhibition of apoptosis and its role in normal cell division have been described in the literature and will be briefly summarized here.

Survivin's function at cell division is to target members of the chromosome passenger complex (CPC) to kinetochores. This complex, comprised of inner centromere protein (INCENP), aurora-B kinase, and borealin, has important functions for normal mitotic processes, including chromosome condensation, chromosome congression, sister chromatid separation, and cytokinesis (260, 261). Functional CPC is responsible for the attachment of chromosome kinetochores to mitotic spindles (262). Defects in this pathway lead to mitotic defects, such as aneuploidy. Aside from CPC survivin, a distinct pool of microtubule-associated survivin exists, which functions to enhance spindle formation through increased microtubule stability (263). Microtubule-associated survivin is phosphorylated by cyclin-dependent $p34^{cdc2}$-cyclin B1 at G2/M, which has been determined to be

essential to maintain microtubule stability and inhibit apoptosis of mitotic cells (264).

Survivin's anti-apoptosis has been attributed largely to its ability to interact with cytosolic proteins, specifically those involved in mitochondrial-associated cell death. Research has demonstrated the interaction of survivin with caspases, the family of cysteine proteases responsible for cytochrome *c*-induced apoptosis, prevents the formation of the apoptosome and downstream apoptotic pathways (265-267). One such interaction is the ability of survivin bound to hepatitis B X-interacting protein (HBXIP), to directly inhibit pro-caspase-9 (268). Activated caspase-9 is required to activate effector caspases-3 and caspase-7, which execute mitochondrial-induced apoptosis (269). Thus, the disruption of this pathway prevents apoptosis and cell death. A separate pool of survivin in the mitochondria has been identified in tumor cells that, upon apoptotic stimuli, is expelled to the cytoplasm (270). XIAP (X-linked IAP) binds this pool of survivin, and antagonizes the effect of caspase-3 and 9 that, again, prevents cytochrome *c*-mediated apoptosis (270, 271). The second mitochondria-derived activator of caspase (SMAC, also called DIABLO) is also released by the mitochondria in response to apoptotic stimuli. This protein is capable of inhibiting XIAP, thereby preserving caspase activity and cell death (272). Survivin is capable of modulating the activity of SMAC/DIABLO. First, mitochondrial survivin delays the release of SMAC from the mitochondria after apoptotic stimuli (273). Second, cytosolic survivin can bind and sequester SMAC away from XIAP, thus allowing XIAP/survivin to antagonize caspases 3 and 9 (274).

The transformation of survivin from an undetectable protein in differentiated tissue to a major player in cancer cells can be explained by the regulators of survivin's expression, which is under the control of the *BIRC5* promoter (259). The

tumor suppressor p53 is stabilized by DNA damage, which then represses *BIRC5* transcription (275). However, the loss of p53 function common in cancer may contribute to the over-expression and improved cell survival seen with survivin over-expression (112, 276). Oncogenic factors that bind to the *BIRC5* promoter and induce the expression of survivin have been identified, such as transcription factor 4 (TCF4) and signal transduction and activator of transcription 3 (STAT3) (277-279). It is this dysregulation and over-expression of survivin in tumor tissues that presents a unique target for cancer therapy, and is exceptionally well suited as a first target for our proposed targeted protein degradation system.

Proteasomal Degradation of Survivin

This ubiquitin-proteasome pathway degrades survivin, with ubiquitination occurring on multiple lysine residues (280). The exact survivin degradation pathway has not been fully elucidated, but its mechanism appears to be unique from other inhibitors of apoptosis, and may be dependent upon a XIAP-XAF1 complex, where XAF1 (XIAP-associated factor 1) activates the E3 domain of XIAP, which then ubiquitinates survivin and sends it to the degradation pathway (281, 282). Survivin's half-life is cell line-, and cell cycle- dependent, and is typically short in embryonic cells (around 30 minutes). However, posttranslational processes involved in tumor cells increase survivin's half-life, as evidenced by a reported half-life of 10 hours in MCF-7 human breast cancer cells (283). The increased half-life of survivin in a tumor cell line highlights the potential to directly reduce its half-life by inducing its degradation.

The Controlled Degradation of Survivin

The presence of survivin in tumor tissues provides a heightened survival threshold, increases cellular proliferation, and decreases responsiveness to cytotoxic agents (111). Specifically inducing the degradation of over-expressed survivin should reduce the survival threshold, decrease proliferation, and increase responsiveness to cytotoxic agents.

The controlled proteasomal degradation of p53 was previously described, but we propose that protargs can be created with diverse ubiquitin-interacting domains (UID). Here, we propose a protarg with full-length estrogen receptor (ER), whose degradation can be controlled by the addition of fulvestrant. With a survivin binding domain fused to ER, we hypothesized that endogenous survivin will also be degraded due to its binding to the protarg via the survivin-binding domain (SBD).

To degrade endogenous survivin, these protargs will promote survivin degradation *in trans*. In other words, the interaction of the protarg with E3 ubiquitin ligases will also induce the ubiquitination of the survivin molecule to which it has bound. Evidence of *in trans* ubiquitination and degradation of proteins can be found in the MDM2/p53 interaction previously described (113, 284). Investigators have found that removal of the MDM2 binding domain from p53 still allowed the ubiquitination and degradation of that mutant by MDM2—through oligomerization with other wild-type p53 molecules still capable of the MDM2 interaction (285).

Native survivin (GenBank accession AK311917) exists as a homodimer (286, 287), and our protargs will utilize this dimeric arrangement by including full-length survivin, allowing for protarg/survivin binding. As survivin is an inhibitor of apoptosis, the introduction of a wild-type survivin protarg fusion may confer an

oncogenic phenotype. Work by the Altieri group has shown that mutating survivin threonine 34 to alanine (located in the baculovirus IAP repeat domain) results in a dominant negative IAP phenotype (117, 264, 288). There is a phosphorylation event on threonine 34 cyclin-dependent $p34^{cdc2}$-cyclin B1 at G2/M, and this has been determined to be crucial for survivin's anti-apoptosis (264). Removal of this threonine prevents survivin's inhibition of apoptosis. By using this mutation, the introduction of our protarg into a cell should not produce deleterious effects. Plasmids will be constructed by previously described methods such that the protarg follows the UID – survivin binding domain – EGFP arrangement.

Current Survivin-Targeted Therapies

The high specificity for survivin in cancer has garnered attention from the drug discovery community, and a small portfolio of clinically tested therapies has been produced (289). The nodal nature of survivin provides a unique target in cancer cells that has the ability to bypass the vast molecular heterogeneity, particularly in breast cancer (112). The traditional paradigm of druggable proteins is that suitable targets are those expressed on cell surfaces, contain catalytic activity, or have structural regions appropriate for small molecule binding (290). However, crystallographic data and studies of protein localization have not demonstrated these characteristics (286, 287). Thus nontraditional approaches to therapy have been pursued, such as antisense molecules, vaccines, and transcriptional repressors (112, 289, 291). In breast cancer patients, two phase I studies are currently in progress testing vaccine-based therapies in metastatic breast cancer (289). However, in a separate Japanese study of a survivin variant peptide vaccine, no beneficial clinical response was observed (292).

Aside from breast cancer, studies on survivin targeted therapy have been conducted and are in phase I or II. The survivin antisense molecule LY2181308 has completed phase I trials, and is currently undergoing phase II studies in refractory acute myeloid leukemia and hormone refractory prostate cancer (293). While no data has been published on the phase I studies, results from xenograft models demonstrated inhibition of tumor cell proliferation, increased sensitivity to cytotoxic agents or radiation, spontaneous apoptosis, and reduced tumor growth (294). Two transcriptional repressors have also been studied: EM-1421 and YM155. EM-1421 has completed phase I studies, and is undergoing phase II studies in a wide range of tumors including refractory leukemias and solid tumors (295). YM155 has completed phase I and phase II studies in refractory solid tumors, and had manageable side effects, potentially unrelated to treatment, but has produced only modest results (296-298).

Nonclinical work has also been done with ribozyme RNA interference (116), gene therapy with dominant-negative survivin mutants C84A and T34A (266, 299), and a survivin/HSP90 antagonist (300). The survivin promoter has been proposed for tumor-specific transcription of cytotoxic genes (301-303), and may prove useful for future studies of survivin protarg expression in tumor cells.

The small portfolio of survivin therapies, while limited, has shown tolerability, but the efficacy is yet to be demonstrated as phase II and, potentially, phase III studies are published. While clinical data are lacking, xenograft models have consistently shown that disrupting the survivin network prohibits tumor cell survival and leads to tumor shrinkage (304).

Survivin and Breast Cancer

In women, breast cancer is the leading cancer diagnosis, accounting for 29% of new diagnoses (305). It is the second leading cause of cancer deaths in women, second only to lung cancer. Currently, a woman has a one in eight chance (12%) that she will develop an invasive breast cancer at any point in her life (306). The most recent statistics show an overall 5-year survival rate of 89% (307), but advanced tumors result in shorter recurrence-free and overall survival times (308). While the overall survival statistics are promising, the heterogeneity of the disease leads to difficulty in personalized treatment, and aggressive advanced tumors are lacking in safe and effective treatments.

Molecular markers often serve as targets for clinical management of the disease. For example, an estrogen receptor positive tumor can be effectively treated with estrogen antagonists, such as tamoxifen, reducing the risk of cancer death by 30% each year (309). Tumors over-expressing HER2/neu direct therapy toward trastuzumab-containing regimens, which reduces the rate of disease recurrence by half (310, 311). Advanced tumors lacking druggable molecular markers become more resistant to conventional antineoplastic treatment, such as doxorubicin, cyclophosphamide, and paclitaxel, leaving patients with few successful treatment options.

In the past few years, the prognostic importance of the inhibitor of apoptosis survivin has been implicated in breast cancer (312-315). In studies of breast cancer patients with grade I-III tumors, survivin over-expression was determined to be a poor prognostic factor. Survivin expression occurred in 60-90% of these patients. High survivin expression levels (set at > 1.4 ng/mg total protein) was associated with nodal positivity, worse disease-free survival, and worse overall survival rates (312).

Survivin was also associated with estrogen and progesterone receptor negative status. Recently, an acetylated form of survivin (acetyl129) was evaluated in breast cancers, and it was found that the acetylated form was associated with a favorable outcome in luminal-type tumors, whereas unacetylated survivin was associated with a poor outcome in the basal phenotype (316). This evidence, along with studies linking survivin with clinically aggressive disease including tamoxifen-treated recurrent diseases (317), antineoplastic resistance (277), and metastasis (318), brings survivin to the forefront in searching for a new target among the complex molecular subtypes of breast cancer.

Statement of Objectives

The controlled localization of proteins may be potentially harnessed for therapeutic use. Three such localization-controlled constructs are discussed: cytoplasm-to-nucleus protein switch, ER LBD induced aggregation at the cytoskeleton, and proteasome-targeted p53 and ER constructs. The following hypotheses directed the scientific pursuit of localization controllable proteins:

Hypothesis 1: Altering the nuclear export signal, nuclear localization signal, or ligand binding domain will alter the amount of protein translocating from the cytoplasm to the nucleus in response to ligand.

Aim: To optimize the amount of protein translocation of a protein switch by altering the nuclear export and localization signals and combining them with various ligand-binding domains.

Hypothesis 2: A fusion protein containing the estrogen receptor ligand-binding domain can be sequestered in the insoluble cytoskeletal fraction by adding the antiestrogen drug fulvestrant.

Aim: To fuse a protein of interest to the estrogen receptor ligand-binding domain so that when the drug fulvestrant is added, the protein is sequestered in the insoluble cytoskeletal fraction where its activity is abolished. The degree of localization control is examined by adding signal sequences to direct subcompartment specificity. Also, we aimed to test the ability of sequestering a functioning peptide to the cytoskeleton in order to abolish its activity.

Hypothesis 3: Controlling a protein's interaction with an E3 ubiquitin ligase can regulate its targeting to the ubiquitin-proteasome pathway.

Aim: To create proteasomal-targeted proteins capable of controlled proteasomal degradation with p53 or full-length ER.

These hypotheses and aims are discussed in the chapters that follow. Chapter 2 reviews controlling protein compartmentalization to overcome disease, as published in *Pharmaceutical Research* (1). Chapter 3 describes Hypothesis 1. Chapter 4 describes the use of ERLBD to sequester proteins in the insoluble fraction, as published in *Pharmaceutical Research* (319). In Chapter 5, the p53 protarg and its ability to target the ubiquitin-proteasome pathway is presented as it was submitted to *Molecular Pharmaceutics.* Finally, Chapter 6 describes the use of full-length ER as a protein capable of proteasomal-targeting.

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CHAPTER 2

CONTROLLING PROTEIN COMPARTMENTALIZATION TO OVERCOME DISEASE

Abstract

Over the past decade, considerable progress has been made to improve our understanding of the intracellular transport of proteins. Mechanisms of nuclear import and export involving classical receptors have been studied. Signal sequences required for directing a protein molecule to a specific cellular compartment have been defined. Knowledge of subcellular trafficking of proteins has also increased our understanding of diseases caused due to mislocalization of proteins. A specific protein on deviating from its native cellular compartment may result in disease due to loss of its normal functioning and aberrant activity in the "wrong" compartment. Mislocalization of proteins results in diseases that range from metabolic disorders to cancer. In this review we discuss some of the diseases caused due to mislocalization. We further focus on application of nucleocytoplasmic transport to drug delivery. Various rationales to treat diseases by exploiting intracellular transport machinery

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JRD wrote the introduction, import and export sections, and created the figures therein, and assisted with the sections on specific diseases. MK wrote the section on specific diseases, and CSL wrote the applications section.

have been proposed. Although the pathways for intracellular movement of proteins have been defined, these have not been adequately utilized for management of diseases involving mislocalized proteins. This review stresses the need for designingdrug delivery systems utilizing these mechanisms as this area is least exploited but offers great potential.

Introduction

The discovery of the "Signal Hypothesis" led Gunter Blobel to win the 1999 Nobel Prize for Physiology or Medicine (1-4). Blobel discovered that protein "zip codes" exist for directing proteins to subcellular compartments such as the nucleus, cytoplasm, mitochondria, endoplasmic reticulum, lysosomes and endosomes, peroxisomes, golgi, and nucleolus (Table 2.1). Proteins need to be directed to their proper cellular compartments in order to perform their necessary functions. For example, most transcription factors need to be in the nucleus to promote gene expression. Some proteins, such as the glucocorticoid receptor, may start in one compartment (cytoplasm), and move to another compartment (nucleus) in response to a stimulus (ligand).

Spotlight on Nucleocytoplasmic Shuttling:
from Basics to Therapeutic Potential

Regulation via Subcellular Compartmentation
and the Nuclear Pore Complex

On the cellular level, macromolecular traffic between the interphase eukaryotic nucleus and cytoplasm of cells represents a highly sophisticated level of cellular regulation that requires effective and selective transport machinery. Protein

Table 2.1

Signal Sequences for Subcellular Compartments

Targeted subcellular compartment	Signal(s)	References
Nucleus	Monopartite PKKKRKV; Bipartite KRPAATKKAGQAKKKKLDK	(4, 6) (6)
Cytoplasm (export from nucleus)	LX(1-3) LX(2-3) LXJ (L= Leu X= spacer J= Leu, Val or Ile)	(7-10)
Mitochondria	***M*LSL*R*QSI*R*FF*K*PAT*R*TL** (amphipathic a helix) +chg residues on one side (italics); non-polar residues on the other side (bold)	(11, 12)
Endoplasmic Reticulum (return to ER)	KDEL at C-terminus	(13, 14)
Lysosomes and Endosomes	Tyrosine-based sorting signals: NPXY or YXX Di-leucine-based [DE]XXXL[LI] and DXXLL consensus motifs	(15)
Peroxisomes	SKL at C-terminus	(11, 16)
Trans-golgi network	di-leucine motif followed by two acidic clusters: LL*EDD*S*DEEED* (acidic clusters italicized)	(17)
Nucleolus	Basic stretches of aa's such as RRRANNRRR KKKMKKHKNKSEAKKRKI	(18-20)

activity can be regulated by selective import and export (5, 21), a “compartmentation allows regulation of key cellular events” (5). Likewise, regulation of nuclear import provides a mechanism for control. Continuing with our example, transcription factors can be kept in the cytoplasm until a signal triggers their import into the nucleus where they can interact with DNA/genes (22). Import and export of proteins occurs through the nuclear pore complex (NPC) (Figure 2.1) (6).

The approximately 125 MDa NPC perforates both lipid bilayers of the nuclear envelope (23), forming the exclusive site by which ions, small molecules, and macromolecules must pass. High resolution electron microscopic studies of Saccharomyces cerevisiae, Xenopus laevis, and rat liver cells have revealed an overall tripartite structure: cytoplasmic filaments, a central transporter, and a nuclear basket, all conferring 8-fold rotational symmetry in the plane normal to the membrane (24) [reviewed in (25-27)]. Eight long cytoplasmic filaments, connected proximally by a coaxial ring, radiate away from plane of the membrane, accounting for one-fourth of the total NPC mass. These projections contain so-called FG repeats, having amino acid repeats in the form of FXFG, GLFG, or FG (where F is phenylalanine, G is glycine, L is leucine, and X is any), which interact with karyopharins (family of transport receptors), and deflect non- shuttling proteins (28-31). The cylindrical intramembrane transporter is surrounded by eight spokes which anchor it to the membrane, collectively accounting for nearly half of the entire mass. Central pores allow the passive diffusion of molecules less than 9 nm in diameter (~40 kDa) via an aqueous channel (32), while a gated, iris-like movement allows the selective active transport of macromolecules of up to 39 nm in diameter (40-60 kDa)

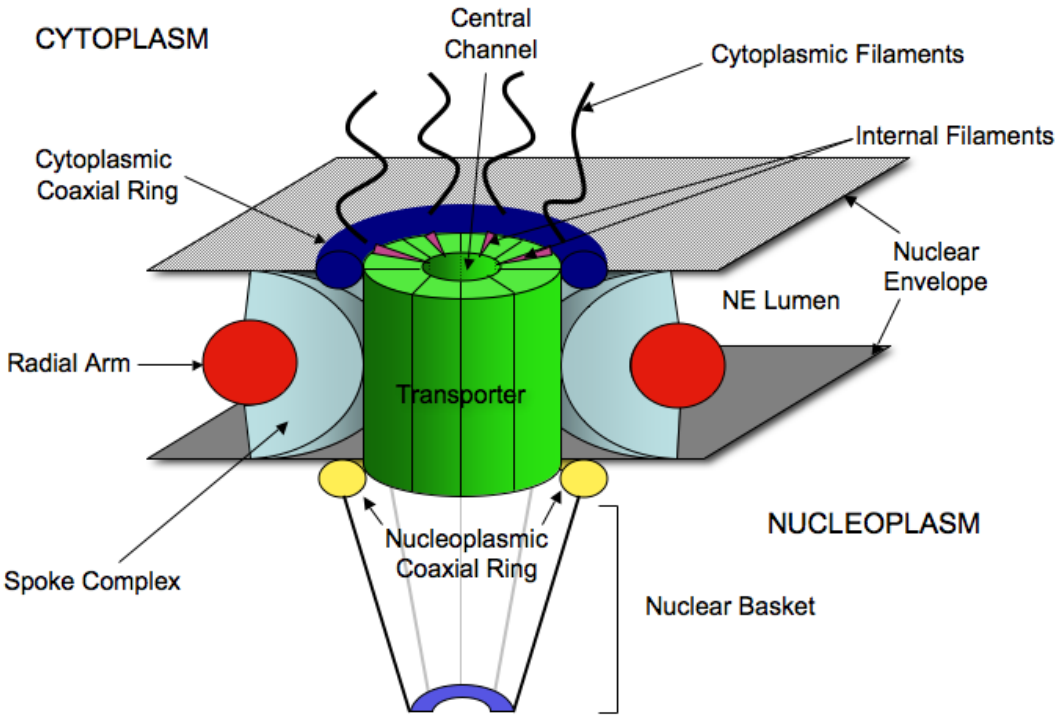

Figure 2.1. The nuclear pore complex. Half of the complex is cut away, except with the central transporter which is shown complete. NPCs exhibit octagonal symmetry about the plane perpendicular to the nuclear envelope. Eight filaments stretch into the cytoplasm, directly interacting with the karyopharin family of transporters. Connecting the filaments is a coaxial ring. Within the double membrane, a central transporter with eight repeating proteins is surrounded by a spoke complex. Nucleoplasmic filaments project into the nucleoplasm and connect distally by a ring structure, forming a basket. The NPC is the sole location for translocation across the nucleus' double membrane.

(33, 34). On the nucleoplasmic side, eight long filaments project away from the membrane and connect distally by a ring, forming a basket. The specific proteins that make up the NPC are called nucleoporins (nups). Due to the highly symmetric nature of the structure, only about 100 nups constitute the complex, but many are present in repeats of 8-16 (26, 27). Nups and NPCs, however, are not as simple as a three-part structure only involved in nucleocytoplasmic shuttling. Several nups have been shown to shuttle within the cyto- and nucleoplasmic portions of the complex (35-38). Further, nups have been implicated in spindle and kinetochore assembly (39-41), and chromatin organization and transcriptional activation (42).

Nuclear Import and Nuclear Localization Signals

Nuclear import and export utilize non-universal pathways that include many specific proteins. Discussed in this paper are only generalized cycles established for the classical import and export signals. For more detailed reviews, see Görlich et al. (5), Weis (43), Pemberton et al. (44), and Macara (45). (Fig. 2.2) diagrams the generalized import pathway for classical nuclear localization signal-carrying proteins. Initiation of import begins in the cytoplasm, with the recognition of importin α to a nuclear localization signal (NLS). Importin α itself cannot interact with the NPC for translocation, so an adapter protein—importin β—is needed. This three-protein complex translocates across the nuclear envelope into the nucleoplasm. RanGTP binds to the amino- terminus of importin β, resulting in release of the cargo- importin α duplex. Subsequently, the cargo is available to function in the nucleoplasm, while importin α and β are recycled to the cytoplasm through separate RanGTP-dependent events. Back in the cytoplasm, RanGTP is hydrolyzed by

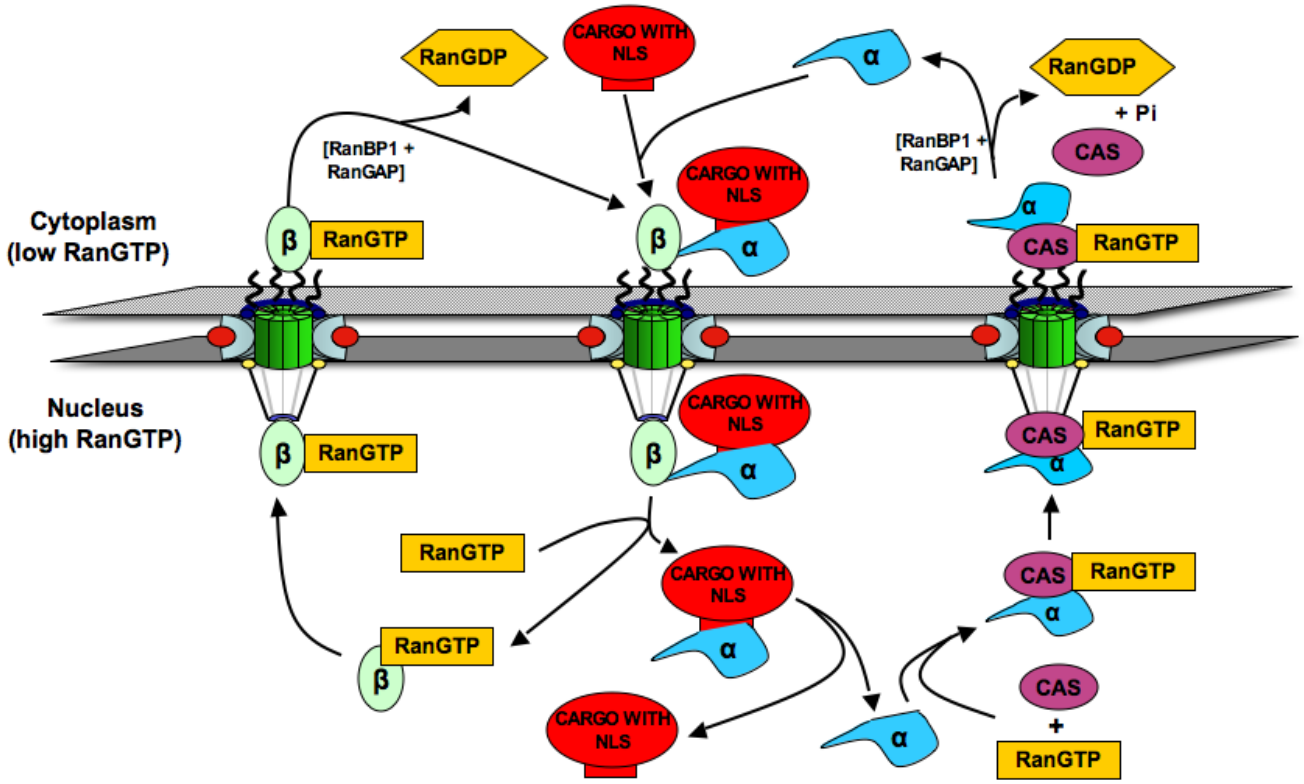

Figure 2.2. Import of proteins carrying a classical NLS. (a) Initiation of import begins in the cytoplasm, with the recognition of importin α to a nuclear localization signal (NLS). Importin β is needed to translocate the complex across the envelope. (b) Once in the nucleoplasm, RanGTP binds to the amino-terminus of importin β, resulting in release of the cargo-importin α duplex. Subsequently, the cargo is available to function in the nucleoplasm, while importin α and β are recycled to the cytoplasm. (c) CAS and RanGTP are needed to shuttle importin α, (d) while only RanGTP is needed for importin β. Back in the cytoplasm, (e) each RanGTP is hydrolyzed by RanGAP to RanGDP, releasing bound karyopharins, where another round of import may take place. Therefore, two GTPs are needed for import of one protein cargo. Adapted from Görlich et al. (5).

RanGAP to RanGDP, releasing bound karyopharins, where another round of import may take place.

Nuclear localization signals (NLSs) were discovered decades ago as the means for active transport of larger macromolecules into the nucleus (46). Proteins containing classical nuclear localization sequences (NLSs) are imported into the nucleus by the importin α/β heterodimer (47). Importin α contains the NLS binding site, whereas importin β mediates the translocation through the nuclear pore (5, 48). Ran, a small GTPase which converts from RanGDP to RanGTP in the nucleus, plays a critical role in both import and export. Its nucleotide state is determined by regulators which have opposite localization, and thus a gradient across the membrane can be formed (49). Importins respond to the RanGTP/GDP gradient, approximately 200-fold (50), which is a driving force for the transport of cargo proteins from the cytoplasm to the nucleus (5). Classical NLSs are either a single stretch (monopartite) of basic amino acids like the one from SV40 large T antigen (PKKKRKV; critical residues underlined) (46, 51), or bipartite (two short sequences with spacer) basic amino acid stretches like the one from nucleoplasmin (KRPAATKKAGQAKKKKLDK) (6). With NLSs, flanking sequences often are important for localization as well (51). Additional tripartite signals have been identified in some steroid hormone receptors, encompassing a SV-40 monopartite signal within them (52). The human PR, like other steroid receptors, contains a constitutively active classical NLS (at position 637-645, RKFKKKFNK) and also contains a non-classical NLSs that is ligand inducible (NLS_i) (53-55). This non-classical NLS has no apparent common motif compared to classical NLSs, although part of it encodes the 2nd zinc finger of the DNA binding domain (DBD) of PR. PR's NLS_i aa sequence is RAMEGQHNYLCAGRNDCIVDKIRRKNCPACRLRKCCQAG-

MVLGG (from position 593-636). Underlined sequences containing basic amino acids R and K likely interact with importin machinery (56). It has not been fully elucidated what import receptor(s) interact with NLS_i, but presumably they must still enter the nucleus via the NPC (55).

Nuclear Export and Nuclear Export Signals

Importins and exportins both interact with RanGTP by an amino-terminal domain, but have opposite effects on protein trafficking. During import, proteins are released upon RanGTP binding in the nucleus. Conversely, in export, cargo proteins only bind to transporters in the presence of RanGTP (Figure 2.3). Upon binding, the cargo/exporter/RanGTP complex translocates through to the cytoplasm where RanGAP catalyzes the hydrolysis of a phosphate bond. RanGDP no longer binds to the exporter, and the whole complex dissociates. RanGDP is imported to the nucleus by NTF2, preserving the Ran-nucleotide gradient. RCC1 in the nucleus exchanges GDP for GTP, and now the cycle is ready for another turn (5, 34, 57, 58). CRM1 (exportin 1) is the classical export receptor for cargos proteins containing leucine rich cargos NESs (5, 59). The formation of a CRM1-cargo complex requires RanGTP. Export by CRM1 is saturable (5, 60, 61). Another exporter is calreticulin (CRT) (62) which may function as an alternative exporter for proteins (such as steroid receptors) with leucine rich NESs (63-66). CRT interacts with cargo in a RanGTP dependent manner. However, Walther *et al*. have suggested that CRT can only export steroid receptors "under stress conditions" (67). Classical NESs are ~10 amino acid sequences with hydrophobic residues, including leucine. We have noted

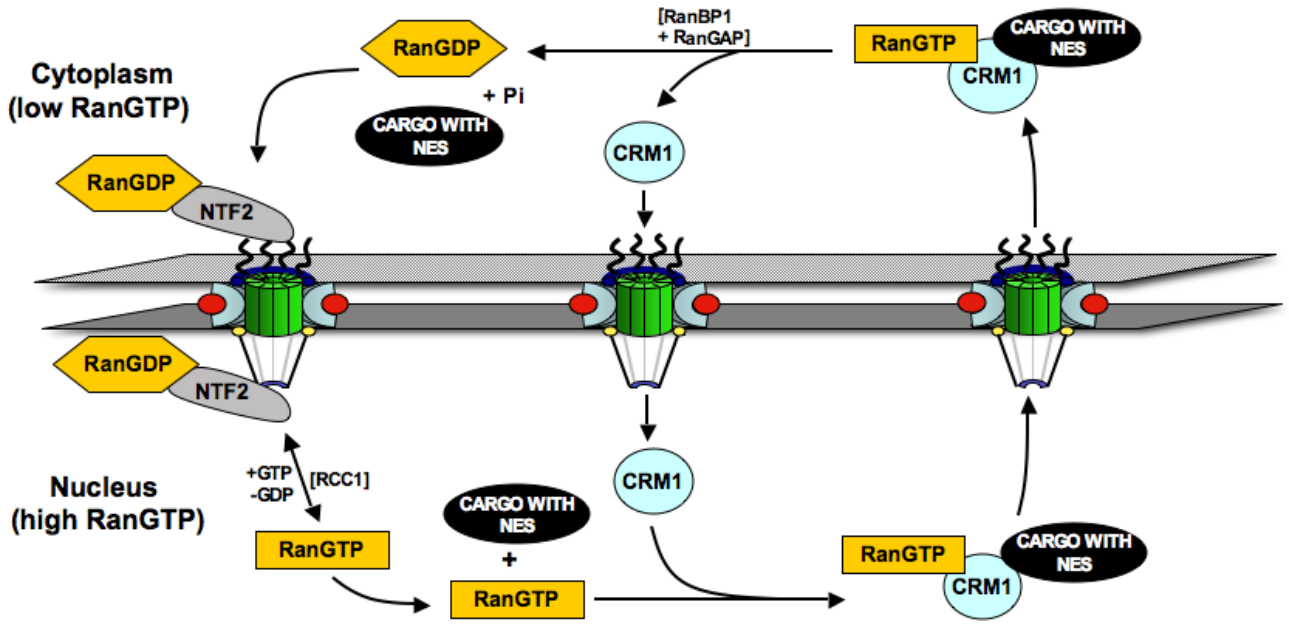

Figure 2.3. Classical export of proteins from the nucleus. (a) Export beings with CRM1, the main export protein, binding a nuclear export signal (NES) on a protein cargo. CRM1 can only bind cargo in the presence of RanGTP. Once complexed, translocation to the cytoplasm takes place. (b) RanGAP and RanBP1 catalyze the hydrolysis of a phosphate bond, creating RanGDP and resulting in the dissociation of CRM1 with its cargo. (c) RanGDP is shuttled back to the nucleus via NTF2 and (d) GDP is exchanged for GTP by RCC1. Adapted from Görlich et al. (5).

a common consensus NES is LX(1-3) LX(2-3) LXJ where L= Leu X= spacer J= Leu, Val or Ile (7), in agreement with others (8-10).

Import, Export, and Other Signals: Application to Drug Delivery

Using signal sequences for precise drug delivery is attractive due to the potential for drugs to be targeted to specific cellular compartments. Peptide NESs have been attached to oligonucleotides for delivery to their site of action, the cytoplasm (68), while NLSs can be used to enhance non-viral gene transfer (69). Similarly, the M9 shuttling (import/export) signal sequence attached to cationic peptides have been used as a delivery system for plasmid DNA (70). An excellent review summarizes the nuclear import, export, and shuttling signals, and their application to drug delivery (71). In all of the examples mentioned here, signals have been used unidirectionally to target a drug--be it an oligonucleotide, polymer or plasmid--to a final compartment.

Known signal sequences are in part defined by their ability to confer localization to a particular compartment, even when taken out of context of the whole protein. Hodel *et al*. show that various NLSs linked to GFP can be used as a model system to test nuclear import (6), while we have shown the same for NESs to test nuclear export (7).

When proteins mislocalize to the wrong compartment, disease can occur (Table 2.2). Many types of cancers arise from mislocalized proteins (72). For example, tumor suppressors that mislocalize to the cytoplasm in cancer cells include p53 (72, 73) and INI1/hSNF5 (74, 75). For p53, the tumor suppressor activity of this protein is suppressed due to its inability to localize in the nucleus. It has been

Table 2.2

Proteins Whose Mislocalization Causes Disease

Disease caused by mislocalization	Type of Protein	Name of Protein(s)	Normal localization	Mislocalization	Refs
Various types of cancers	Tumor suppressor	p53	Nucleus	Cytoplasm	(73, 74)
Various types of cancers	Member of the beta-galactoside binding protein family	Galectin-3	Nucleus	Cytoplasmic when phosphorylated	(78)
Various types of cancers	Forkhead family of transcription factors	FOXO	Nucleus	Cytoplasm	(72, 79)
Various types of cancers	Transcription factor	NF-kB	Cytoplasm	Nucleus	(72, 80, 81)
Breast cancer	Tumor suppressors	BARD1 and BRCA1	Cytoplasm	Dimerization masks NES and causes nuclear accumulation	(82-84)
Malignant rhabdoid tumors	Tumor suppressor	INI1/hSNF5	Nucleus	Cytoplasm	(72, 74, 75)
Cisplatin drug resistance	Multidrug resistant proteins	MRP1	Recycled to plasma membrane	Cytoplasmic accumulation	(85)

Table 2.2 Continued

Acute Myelogenous Leukemia (AML)	Cyclin-dependent kinase inhibitor	p27Kip1	Nucleus	Cytoplasm	(86)
AML	Multifunctional nucleocytoplasmic shuttling protein	Nucleophos-min	Nucleolus	Cytoplasm	(86)
Colorectal cancer	Multifunctional protein; part of the Wnt signal transduction pathway	b-catenin	Cytoplasm	Nucleus	(72, 88)
Chronic myelogenous leukemia (CML)	Bcr- a GTPase activating protein; Abl- a tyrosine kinase oncogene	Bcr-Abl	Bcr alone is cytoplasmic; Abl is nuclear and cytoplasmic	Cytoplasm	(89)
CML	Cell cycle inhibitor	$p21^{WAF1}$	Nucleus	Cytoplasm	(90)
Retinitis pigmentosa (Class I deletion mutant, Q344ter C-term. deletion)	G-protein coupled receptor (GPCR)	rhodopsin	Membrane sacs within the rod	Plasma membrane of photoreceptor cell body	(91, 92)

Table 2.2 Continued

Nephrogenic diabetes insipidus, X-linked (Class I and II)	GPCR	Vasopressin V_2 receptor	Cell surface	Majority of mutations lead to intracellular retention	(92, 93)
Familial hypercholesterolemia (Class II and IV)	GPCR	LDL receptor	Cell surface and internalized	Class II- retained in ER; Class IV- defective internalization	(94)
Cystic fibrosis (some forms)	GPCR	CTFR	Cell surface	Endoplasmic reticulum	(92, 95)
Schizophrenia	GPCR	Dopamine D3 receptor (mutant)	Plasma membrane	Intracellular compartment	(96)
Primary dystonia (DYT1)	*A*TPase *a*ssociated with different cellular *a*ctivities protein family	Torsin A	ER luminal protein	Nuclear envelope	(97)
Primary Hyperoxaluria	Aminotransferase	Alanine:glyoxylate amino-transferase	Peroxisome	Mitochondria	(98)
Stargardt-like macular degeneration	Fatty acid elongase family	ELOVL4	ER	Cytoplasm (5bp deletion mutant)	(99)

reported that mutations of p53 exist in half of all human cancers, leading to inactivation (72, 76). On the other end of the spectrum, the nuclear localization of oncoprotein Ras may be important in oncogenic activation as well (77). Similarly, the mislocalization of cell cycle inhibitors can be detrimental. Relocalization of a cell cycle inhibitor to the "wrong" compartment can lead to tumor progression. This occurs with the cell cycle inhibitor $p21^{WAF-1}$ which normally localizes in the nucleus where it exerts its inhibitory action. Mislocalization of this protein to the cytoplasm leads to tumor progression (90).

G-protein coupled receptors (GPCRs) can also mislocalize and cause disease (92). Specific examples include mislocalized rhodopsin which can lead to retinitis pigmentosa, and mistargeted vasopressin V_2 receptor leading to nephrogenic diabetes insipidus. Finally, defects in trafficking or localization of the LDL receptor can cause familial hypercholesterolemia (94), and mislocalized CTFR leads to cystic fibrosis (95). See Table 2.2 for other examples of diseases caused by mislocalization.

Specific Diseases Induced by Mislocalized Proteins

Breast Cancer: BARD1 and BRCA1

The breast cancer-associated protein BARD1 (thought to be a tumor suppressor) in itself contains proapoptotic activity when in the cytoplasm. BARD1 contains a nuclear export signal that allows compartmentalization into the cytoplasm. However, the breast and ovarian cancer susceptibility protein 1 (BRCA1), a known tumor suppressor, can modulate BARD1 apoptotic activity by binding to BARD1 and blocking its export signal. This results in a marked reduction of BARD1 apoptotic activity. Interestingly, it was found that "BRCA1 and BARD1 regulate the

subcellular localization of one another through the reciprocal masking of their respective nuclear export signals" (83). However,

when BARD1 and BRCA1 dimerize, their resulting nuclear compartmentalization leads to events that promote cancer (DNA repair activity and prolonged cell survival) (82-84) instead of apoptosis. In this situation, the delicate balance between nuclear import and export dramatically alters the function of proteins, and this is controlled by the cell.

Schizophrenia and the Dopamine D3 Receptor

In some forms of schizophrenia, a mutant dopamine D3 receptor has been implicated. This mutant dopamine receptor (D3nf) physically can interact with the normal version of the D3 receptor and causes mislocalization of D3 from the plasma membrane to an intracellular compartment, "a finding that may have significance in the etiology of schizophrenia" (96). In these studies, it was suggested that D3nf may act as a dominant-negative regulator of D3 receptor activity, forcing wtD3 to mislocalize thereby resulting in disease.

Leukemia and BCR-ABL

BCR-ABL protein can be converted from an oncogene to an apoptotic factor if it can be sent to the nucleus (89). BCR-ABL is an oncogene implicated in chronic myeloid leukemia and Philadelphia chromosome positive acute lymphoblastic leukemia. BCR-ABL proteins have abnormal tyrosine kinase activity that leads to oncogenesis (100). BCR-ABL proteins are oncogenic in the cytoplasmic compartment only, and must multimerize in order to be active. BCR-ABL, when directed to the nucleus, indeed becomes apoptotic (89), so this is a possible new way to treat CML.

These same authors found that inhibiting export of BCR-ABL in cell culture using leptomycin B (LMB), a general export inhibitor (causing nuclear accumulation of BCR-ABL), resulted in cancer cell apoptosis. However, LMB cannot be used clinically due to neuronal toxicity in phase I clinical trials (89).

Colorectal Cancer and "β-Catenin"

In most colorectal cancers the adenomatous polypsis coli (APC) protein, a tumor suppressor, is mutated (72, 101). In normal cells this protein shuttles between the nucleus and the cytoplasm; however, in colorectal cancer APC is mostly nuclear due to truncations resulting in loss of functional NES. The nuclear export of APC is very critical for its normal functioning in destabilizing and reducing the transcriptional activity of a signal transduction protein β-catenin. Any level of nuclear import of APC reduces the transcriptional activity of β-catenin. The mutations in APC lead to loss of its nuclear export function and inactivation. This is indicated as an early event in tumorigenesis. Loss of APC causes an increase in nuclear β-catenin and its transcription activity, which leads to tumor progression (88).

Targeting Protein Compartmentalization For Therapy

Targeting nucleocytoplasmic shuttling represents an under-explored area for drug delivery, drug targeting, and therapeutics (102, 103). As the mechanisms of mislocalization are being elucidated, there exist more opportunities for drug therapy besides the standard direct inhibition (or activation) of the protein target. There are several ways in which overcoming aberrant localization of protein can be achieved and are outlined as follows.

Blocking General Nuclear Import or Export Machinery

If a disease is caused by mislocalization of a protein to the nucleus (or cytoplasm), inhibitors of general import (or export) could be utilized for treatment. To date, there are no small molecule inhibitors of nuclear import; however, there are several small molecule inhibitors of nuclear export. These are inhibitors of CRM1, the general export receptor. Leptomycin A and B (104), Ratjodone A (105, 106), and PFK050-637 (107) are all small molecule inhibitors of CRM1 that bind to a critical cysteine residue in CRM1 to abrogate nuclear export. Another possible way to inhibit import or export would be to supply peptides encoding a NLS or NES, and competitively inhibit import/export of other cargo containing the NLS/NES signals. Hawiger and coworkers have designed cyclic peptides containing a cell-penetrating motif and a cyclized form of a NLS from NF-kB. This peptide inhibits import of NF-kB (108). The main disadvantages with blocking general nuclear import or export would be toxicity due to general halting of transport of all proteins going into/out of the nucleus, like Leptomycin B (89, 109).

Enhancing Import or Export of Proteins

Proteins given therapeutically may be modified so that import (or export) is enhanced. NLSs and NESs have routinely been added onto proteins to enhance or modify their cellular destination; for an excellent review see Jans *et al.* (71). The main disadvantage with adding proteins with NLSs/NESs is that the patient still expresses the mislocalized protein, and in some cases, the mislocalized protein exhibits a dominant effect over the non-mislocalized protein (96).

Another way to enhance import/export is to increase the interactions of proteins with the components of the NPC. Molecular proteomics methods are being

used to determine interactions of proteins with nucleoporins (110). This information could be used to enhance or block import (or export).

Alteration of Posttranslational Modifications

It is known that protein modifications including phosphorylation, methylation, and ubiquitinylation can lead to altered compartmentalization of proteins in cells (72). Galectin-3 (Gal-3), a novel regulator of apoptosis, translocates from the nucleus to the cytoplasm under apoptotic stimuli. Phosphorylation enhances export of Gal-3 to the cytoplasm. Interestingly, an increase in the cytoplasmic levels of Gal-3 (and loss from the nuclei) correlates with tumor progression (78).

Blocking Import/Export Partner

Many proteins imported/exported via piggyback mechanisms with other proteins (and not via direct interactions with the actual import/export receptors). A well-known example of this is heat shock protein 90 (Hsp90). Hsp90, a molecular chaperone, is known to retain steroid hormone receptors (and other proteins) in the cytoplasm and may be involved in nuclear import of other proteins (111). In another case mitogen activated protein kinase (MAPK) may be involved in regulating nuclear association of the human progesterone receptor (112). The import/export partner itself (Hsp90 or MAPK) likely still interacts with the general import/export machinery.

Controlled Localization Using a Protein Switch

Ligand inducible nuclear import signals and export signals (a bi-directional on/off switch) can be used for the controlled targeting of therapeutic proteins to subcellular compartments. Our work has shown that a model protein (EGFP in this case) can be directed from the cytoplasm to the nucleus using ligand, in a dose-dependent manner (113). The protein is genetically engineered to constitute a nuclear export signal, a nuclear import signal, and a ligand-binding domain (LBD) from a steroid hormone receptor. When the plasmid encoding this protein is transfected into mammalian cells, the protein is expressed mostly in the cytoplasm. When ligand is added, the protein translocates to the nucleus. The rate and extend of nuclear import depends on the dose of ligand and the incubation time. When ligand is removed from the system, the protein exports back to the cytoplasm. The protein can be re-imported again by addition of ligand (113). Since our initial studies, we have optimized the protein switch so that its localization is more cytoplasmic in the absence of ligand, and more of the protein translocates to the nucleus in the presence of ligand (unpublished data). Optimization involves striking the desired balance between nuclear import and export signals of different strengths, and utilizing different steroid hormone receptor LBDs. Such a protein switch could be used in gene therapy, for controlled localization (and function) of a protein such as a transcription factor, or any other type of protein that is active only in the nucleus of the cell. Our first application of the protein switch will be for breast cancer; the nuclear corepressor NCoR will be engineered into the protein switch. NCor is known to repress both progesterone receptor and estrogen receptor mediated transcriptional activity.

Future Directions for Controlling Intracellular Localization of Proteins

As we have outlined in this paper, malfunctioning of nucleo-cytoplasmic transport leading to mislocalization of proteins causes disease. Thus, manipulation of transport pathways could be used to treat disease. Exploiting the intracellular transport pathways provides an exciting area for treatment of such diseases. Specific signal sequences can be utilized in drug delivery systems to improve the efficacy of a drug by increasing the amount of drug reaching its active therapeutic compartment, or to increase compartment accumulation. Based on the knowledge of intracellular transport, new modalities can be designed targeting specific components of the cellular transport machinery. However, for regulating localization of proteins to treat diseases, further investigation of transport pathways that have not yet been clearly understood is warranted.

The classical pathways of nucleo-cytoplasmic trafficking have been well defined. Consensus sequences have been proposed for nuclear import and export signals, and their strengths have been characterized. Nevertheless, it has been studied that certain proteins despite having an import (or export) signal are localized to a different compartment. In some cases this occurs because various proteins mutually regulate their localization by masking and de-masking the localization signal(s). For example, breast-cancer associated protein BRCA1 is involved in masking the export signal of BARD1 via the dimerization domain and keeps it in the nucleus (82, 83, 114). Another such example involves proteins c-Jun and ATF2, involved in cellular transformation, stress response and regulating organ development. Even though ATF2 possesses a nuclear export signal, dimerization with c-Jun in the nucleus prevents its export (115). In another example, the nuclear

export signal of BCR-ABL protein has been suggested to be inactive due to its location in the hydrophobic core of the protein (116). The area of intracellular transport involved with mutual regulation of protein localization by masking or unmasking the localization signal, has been least explored for therapeutic purposes. Designing delivery systems and drugs (small molecules as well as peptides) to mask and unmask the localization signals is a promising avenue for controlling intracellular movement of proteins.

Besides the classical subcellular pathways of transport, there are other mechanisms of protein import/export that have not been completely delineated yet. CRT has been studied to be involved in nuclear export of steroid hormone receptors, independent of the classical CRM1 pathway (63-65). However, some researchers have claimed that CRT acts as an export receptor only under stress conditions (67), and steroid hormone receptors such as glucocorticoid receptor (GR) are indeed exported via a CRM1 dependent transport (117). Another group has proposed that a β helix structure in androgen receptor ligand binding domain acts as a NES (118) though the mechanism for its activity has yet to be delineated. BCR-ABL, a cytoplasmic protein, still localizes to the cytoplasm on removal of its NES (89) which is likely due to interactions between its F-actin binding domain and the cellular cytoskeleton (116). Similar to nuclear export, it appears that multiple pathways exist for nuclear import as well. In our recent study we have shown that the progesterone receptor (PR) translocates to the nucleus on ligand induction even after mutating its constitutive NLS, albeit at a much slower rate (119). GR has also been shown to constitute two NLSs with one acting via the classical importin α pathway, while the other through an agonist specific pathway independent of importin α (117).

Some proteins are transported between two compartments by a facilitator protein or a "piggy-back" mechanism. Localization of various proteins involved in cellular functioning such as p53 (72, 73, 120, 121), β-catenin (72, 88, 101) is regulated by other interacting proteins. Heat-shock protein (Hsp90), one of the most abundant proteins in cells, is touted to be a chaperone protein involved in intracellular movement of various other proteins (122-124). However, the mechanism of transport of Hsp90 is still not defined. Ligand binding domains of steroid receptors are known to affect change in localization of these molecules while in the full length receptor or when taken out of it and induced with a ligand, but no single mechanism explaining change in their localization has been proposed as yet. Thus, there are mechanisms of subcellular trafficking that still need to be elucidated.

Exploring mechanisms of intracellular transport holds the key to devise means to treat a large number of diseases ranging from metabolic disorders to cancer. Studying cellular pathways of macromolecule movement would also aid in a more comprehensive understanding of certain known diseases, resulting in management. Harnessing signal sequences to change localization of proteins and hence their activity, is a novel way of finding cure for many diseases. Designing new delivery systems and drug modalities based on the current knowledge of signal sequences and transport pathways, for treatment and management of diseases, is an exciting new area of molecular pharmaceutics that offers great potential.

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CHAPTER 3

OPTIMIZING THE PROTEIN SWITCH: ALTERING NUCLEAR IMPORT AND EXPORT SIGNALS, AND LIGAND BINDING DOMAIN

Abstract

Ligand regulated localization controllable protein constructs were optimized in this study. Several constructs were made from a classical nuclear export signal (HIV-rev, MAPKK, or progesterone receptor) in combination with a SV40 T-antigen type nuclear import signal. Different ligand binding domains (LBDs from glucocorticoid receptor or progesterone receptor) were also tested for their ability to impart control over localization of proteins. This study was designed to create constructs that are cytoplasmic in the absence of ligand and nuclear in the presence of ligand, and also to regulate the amount of protein translocating to the nucleus on ligand induction. The balance between the strengths of import and export signals was critical for overall localization of proteins. The amount of protein entering the

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MK was the main contributor to this paper, but JRD contributed intellectually with most aspects of the work, and provided technical assistance for microscopy, and image and data analysis. The original publication has been modified here to reflect the experiments that JRD contributed to, and includes additional work done by JRD that cannot be separated from the original paper. SEK contributed intellectually to the import modeling section (not reprinted here), and CSL contributed intellectually to all aspects of the work.

nucleus was also affected by the dose of ligand (10-100nM). However, the overall import characteristics were determined by the strengths of localization signals and the inherent localization properties of the LBD used. This study established that the amount of protein present in a particular compartment could be regulated by the use of localization signals of various strengths. These optimized localization controllable protein constructs can be used to correct for diseases due to aberrant localization of proteins.

Introduction

Intracellular localization of genes and gene products plays an important role in maintaining normal cellular functions. Molecules involved in regulation of the cell cycle–splicing factors, proteasomes, transcription factors, protein kinases, cell cycle inhibitors, chromatin assembly proteins, and numerous other regulatory proteins–undergo changes in intracellular localization during various phases of the cell cycle (1-6). Studies have shown that mislocalization of these proteins and other tumor suppressors can lead to cancer. Over 10 known tumor suppressors are regulated via nucleo-cytoplasmic shuttling, including p53, p73, Beclin, BRCA1, APC, VHL, PML, Smad4, p130, and INI1/HSNF5 (7). Malfunctioning of shuttling can cause disease; conversely, manipulation of shuttling could be utilized to treat disease. In our previous study (Kanwal et al.) (8) a bi-directional protein construct called the "protein switch" was devised, responsive to an external ligand to control intracellular localization of protein. Our current study describes an improved and optimized ligand inducible protein construct that could be used to correct for mislocalization of endogenous proteins involved in certain diseases. The engineered construct containing the mislocalized protein has controlled localization, and can

overcome the aberrant localization of the endogenous malfunctioning protein. Aside from the compartmentalization application presented in this research, ligand induced localization controllable protein constructs can also be used for independent regulation of genes, artificial control of transcription (via transcription factors), engineering of enzymes, gene therapy applications, and biosensor arrays. This ability to regulate and control localization of genes and gene products has the potential to make gene therapy safer and more efficient. It would also expand the range of gene therapy by allowing customized disease management and a greater control over the therapy.

In eukaryotes, DNA replication and RNA synthesis occur in the nucleus, while protein synthesis occurs in the cytoplasm (9). All nuclear proteins, such as histones and transcription factors are synthesized in the cytoplasm and are transported back to the nucleus, whereas, transfer RNA, messenger RNA, and ribosomal RNA are transcribed in the nucleus and are transported to the cytoplasm for translation. Compartmentalization of these macromolecules allows for regulation of these cellular events (10). The double-membrane nuclear envelope separates the contents of the nucleus and cytoplasm (9, 10). Nucleo-cytoplasmic transport of proteins and other macromolecules occurs through nuclear pore complexes present in the nuclear envelope. These pores (diameter ~9nm) allow for passive diffusion of small molecules less than 45-60 kDa in size (9, 11-13). Large macromolecules are transported by an active process utilizing specific transport signals (10) which consist of specific amino acid sequences recognized by different receptors to shuttle proteins to their active compartments.

Signal sequences that are recognized by import receptors and facilitate movement of molecules from the cytoplasm to the nucleus are called nuclear

localization signals (NLS). There are three different classes of NLSs. Classical NLSs are either monopartite, single stretches of basic amino acids (PKKKRK), or bipartite (two short sequences with a spacer) basic amino acid stretches (KRPAATKKAGQAKKKKLDK) (8, 10, 13-16). The mono- and bipartite import signal sequences, from SV40 T-antigen and nucleoplasmin respectively, are considered to be prototypes of classical nuclear localization signals (10, 14, 17). The third class of NLSs, for example, the yeast homeodomain-containing protein Matα-2, has polar residues interspersed with nonpolar residues (MNKIPIKDLLNPQ) (13). The sequences flanking the NLSs are often important for localization (15, 16). In addition to these three classes, tripartite signals (three short sequences with a spacer) have been identified in some steroid hormone receptors, encompassing a SV40 T-antigen like monopartite signal within the signal sequence (18). All NLS sequences are recognized by the importin α/β heterodimer (8, 10, 13). Importin α recognizes the NLS sequence and binds to it via its NLS binding site, while importin β mediates translocation of this importin α-NLS cargo complex through the nuclear pore complex to the nucleus (8-10, 19). SV40 T-antigen type classical NLSs have already been studied for their relative strengths based on their interaction with importin α (16). These classical NLSs with varying import strengths are used in our research. There have been many studies using nuclear import signals to facilitate the movement of macromolecules into the nucleus of cells (20-22). Others have used NLSs have been used to facilitate import of plasmid DNA into the nucleus of cells (23, 24).

Opposing nuclear import, the cell uses leucine-rich nuclear export signals (NES) that actively transport proteins from the nucleus to the cytoplasm. These sequences are recognized by the classical export receptor CRM1 (chromosome region

maintenance), also called exportin 1 (10, 25, 26). Classical NESs are ~ 10 amino acids in length with hydrophobic residues, especially leucines. As a common consensus a NES sequence is LX (1-3) LX (2-3) LXJ where L is leucine, X is a spacer and J is either leucine, valine or isoleucine (8, 27-30). We have shown in our previous study that classical NESs from HIV-rev protein and MAPKK (mitogen activated protein kinase kinase) protein can individually confer export to an enhanced green fluorescent protein (EGFP)-containing construct (8).

Previously we devised localization controllable protein constructs containing a nuclear export signal, a nuclear import/localization signal, and a ligand binding domain to confer responsiveness to an external ligand (8). EGFP was used as a gene of interest to track the localization of a protein construct using fluorescence microscopy (8, 30-34). In the absence of external ligand, the protein construct was mostly present in the cytoplasm due to the dominant effect of the NES. Upon ligand addition, the protein construct translocated to the nucleus via the LBD and the NLS. It was shown that the rate and amount of protein translocating to the nucleus correlated with the dose of ligand (8). The purpose of our current work was to optimize the protein switch to make it more cytoplasmic in the "off" state (unliganded) and more nuclear in the "on" state (with ligand). The utility of the protein switch constructs lies in the ability to keep the attached therapeutic protein in one cellular compartment where it is inactive. Upon addition of ligand, the therapeutic protein can move to another compartment where it has activity. The optimization process leads to improved control of protein location, and better control of protein function. The effect of the critical balance between NES and NLS strengths on the localization of constructs was tested. Based on our previously

published observation indicating that NLSi is a weak import signal (35), the NLSi used in our prototype constructs (8) was replaced with stronger classical NLSs.

The first protein switch used a truncated form of the LBD from the progesterone receptor that is responsive to mifepristone (RU486) (36), and non-responsive to natural agonist progesterone or synthetic agonist R5020. The benefit of an LBD with this ligand response is that naturally occurring hormone in the body would not trigger the translocation of the switch, meaning that it can only be externally controlled. A drawback of this ligand choice is that mifepristone can antagonize wild-type endogenous progesterone and glucocorticoid receptors, and is also used as an abortifacient (8). A protein switch reliant upon a ligand with many natural targets is sub-optimal if this system is to ever reach the clinic, so an LBD that responds to a ligand with fewer side effects or controversies was sought. This chapter focuses on the alternative NES, NLS, and LBD combinations that were examined in the optimization of the protein switch. Table 3.1 shows the various combinations of protein switch constructs discussed in this chapter.

Materials and Methods

Cell Line and Culture Conditions

The murine adenocarcinoma cell line 1471.1 (which does not express endogenous progesterone receptor) was used in this research. Cells were grown as monolayers in 175cm^2 flasks containing DMEM (GIBCO BRL, Grand Island, NY) supplemented with 10% FBS (Hyclone Laboratories, Logan, UT), 1% penicillin-streptomycin (GIBCO BRL), 0.1% gentamicin (0.5mg/ml, Hyclone), and 1% L-glutamine (2nM, Hyclone), and maintained in a 5% CO_2 incubator at 37 ^{o}C.

Table 3.1

Nuclear Export Signals, Nuclear Import Signals, and Ligand Binding Domains Used in this Study

Type of signal/domain	**Amino acid sequence (or numbering)**	**Strength of signal (K_d nM (16), if known)**
Nuclear export signals (NES):		
MAPKK	LQKKLEELEL (28)	strong
HIV	LQLPPLERLTL (28)	medium
PRB NES2	LHDLVKQLHL (30)	n/a
Nuclear import signals (NLS):		
SV40	SPKKKRKVE (16)	9±4 strong
SV40A1	SAKKKRKVE (16)	36±2 medium
SV40A7	SPKKKRKAE (16)	53±4 medium
MycA8	PAAKRVKADE (16)	85±7 medium
SV40A4	SPKKARKVE (16)	335±7 weak
CDC2	GVVYKGRHKTTG (37)	n/a weak
PKC	FVVHKRCHE (37)	n/a weak
NLSi	RAMEGQHNYLCAGRNDCIVDKIR-RKNCPACRLRKCCQAGMVLGG (38)	n/a weak
Ligand binding domains (LBD):		**Ligand**
PR LBD	645-891aa (30)	Mifepristone (MFP)
GR LBD	547-795aa (39)	Dexamethasone (Dex)
Ecr LBD	375-652aa(40)	Muristerone A (MUR)
ER LBD	306-556aa	Hydroxytamoxifen (OHT)

Transient Transfections

Transient transfections of 1471.1 cells were carried out by electroporation as previously defined (8, 30). Transfected cells were plated on a clear cover glass (Corning no.1, 22mm^2) in six well plates or plated into live cell chambers (Lab-tek chamber slide system, 2 ml, Nalge NUNC International, Naperville, IL) containing complete phenol-free DMEM with 10% FBS (charcoal/dextran treated, Hyclone), 1% penicillin streptomycin (100U/ml, GIBCO BRL), 0.1% gentamycin (0.5mg/ml, Hyclone), and 1% L-glutamine (2nM, Hyclone), and maintained in a 5% CO_2 incubator at 37 ^{o}C for 18-24 hours.

Microscopy

Approximately 18-24 hours after electroporation, localization of protein constructs was viewed by fluorescence microscopy (8). An Olympus IX701F inverted fluorescence microscope (Scientific Instrument Company, Aurora, CO) with high-quantity narrow band GFP filter (excitation HQ480/20nm, emission HQ510/20nm, with beam splitter Q4951p) from Chroma Technology (Brattleboro, VT) was used. Cells were photographed at a magnification of 40x using an F-View Monochrome CCD camera. To minimize photobleaching of EGFP chromophore, cells were imaged using neutral density filters at short exposure times of 500ms. An air stream incubator (Nevtek ASI 400, Burnsville, VA) with temperature control was used to maintain the microscope stage at 37^{o}C.

Construction of Plasmids

Different plasmid constructs were created by changing the sequence of NES, NLS and LBD, which hereafter follow the pattern NES-NLS-LBD. The plasmids HIV-NLSi-PRLBD and MAPKK-NLSi-PRLBD, from our previous study (8), were used for construction of new plasmids.

HIV-SV40-PRLBD and MAPKK-SV40-PRLBD were constructed by removing NLSi using restriction enzymes *EcoRI* and *SalI* and replacing it with oligonucleotide 5'- AATTCTAGCCCAAAGAAGAAGAGAAAAGTAGAAG -3' and its complementary strand which encodes the classical NLS from SV40 T-antigen protein (see Table 3.1 for NLS amino acid sequence).

MAPKK-MycA8-PRLBD was constructed by removing NLSi out of MAPKK-NLSi-PRLBD using restriction enzymes *EcoRI* and *SalI* and replacing it with oligonucleotide 5'- AATTCTCCAGCAGCAAAAAGAGTAAAAGCAGACGAAG - 3' and its complementary strand which encodes the classical type NLS sequence from MycA8 protein (see Table 3.1 for NLS amino acid sequence).

HIV-MycA8-PRLBD was constructed by removing MycA8 NLS from MAPKK-MycA8-PRLBD using *EcoRI* and *SalI* and inserting it in HIV-NLSi-PRLBD already digested with *EcoRI* and *SalI* (to remove NLSi).

HIV-SV40A1-PRLBD and HIV-SV40A7-PRLBD were constructed by removing NLSi using *EcoRI* and *SalI* from HIV-NLSi-PRLBD and replacing it with oligonucleotide 5'- AATTCTAGCCCAAAGAAGAAGAGAAAAGTCGAAG - 3' and its complementary strand which encodes SV40A1, a truncated version of classical NLS from SV40 T-ag protein, and oligonucleotide 5'- AATTCTAGCCCAAAGAAGAAG-AGAAAAGCAGA AG - 3' and its complementary strand which encodes SV40A7, a

truncated version of NLS from SV40 T-antigen protein, respectively (16) (see Table 3.1 for NLS amino acid sequence).

MAPKK-CDC-PRLBD, MAPKK-PKC-PRLBD and MAPKK-SV40A4-PRLBD were constructed by removing NLSi using *EcoRI* and *SalI* from MAPKK-NLSi-PRLBD and replacing it with oligonucleotides and their complementary strands encoding NLSs for the respective plasmids. Oligonucleotide 5'-AATTCTGGAGTAGTATACAAAGGA AGACACACAAAACA - 3' and its complementary strand which encodes the NLS from CDC2HS; 5'-AATTCTTTCGTAGTACACAAAAGATGCCACGAAG - 3' and its complementary which strand encodes the NLS from protein kinase C (PKC); and 5'-AATTCTAGCCCAAAAAAAGCAAGAAAAGTAGAAG - 3' and its complementary strand which encodes the truncated NLS (SV40A4) from SV40 T-antigen protein were used to make these constructs (see Table 3.1 for NLS amino acid sequence).

PRNES2-SV40-PRLBD and PRNES2-MycA8-PRLBD were constructed by replacing MAPKK NLS from MAPKK-SV40-PRLBD and MAPKK-MycA8-PRLBD using restriction enzymes *BspEI* and *XhoI* by the oligonucleotide 5' - CCGGACTACAC GACCTAGTAAAACAACTACACCTAGC - 3'and its complementary strand which encodes the classical consensus NES present in progesterone receptor (PRNES2) (8) (see Table 3.1 for NES amino acid sequence).

HIV-MycA8-MycA8-PRLBD was constructed by inserting the oligonucleotide 5'- TCGAGGACCAGCAGCAAAAAGAGTAAAAGCAGACGAAGGG - 3' and its complementary strand which encodes the classical type NLS from MycA8 protein into the construct HIV-MycA8-PRLBD previously digested with *XhoI* and *EcoRI*.

HIV-HIV-MycA8-MycA8-PRLBD was constructed by replacing HIV NES from HIV-MycA8- MycA8-PRLBD with the oligonucleotide 5'- CCGGACTCCAACTG-

CCTCCCTTGGAGCGCCTAACTTGGGAGCACTCCAACTGCCTCCCTTGGAGCGCC TAACTTTGTC - 3' and its complementary strand which encodes the two repeats of the classical consensus NESs from HIV-Rev protein, using enzymes *BspEI* and *XhoI*.

HIV-SV40-GRLBD and HIV-MycA8-GRLBD were constructed by replacing PR LBD with GR LBD (C656G GR LBD (39)) in the constructs HIV-SV40-PRLBD and HIV-MycA8-PRLBD respectively, using restriction enzymes *KpnI* and *BamHI*. The 248 amino acid long C656G GR LBD was taken out of full length GFP-C656G GR by performing PCR using the primers 5' - AGGGTACCCTCACCCCTACCTTG - 3' and 5'- CGCGCGGATCCTTTTTGATGAAACAG - 3'with *KpnI* and *BamHI* ends, respectively.

The plasmid encoding the ecdysone receptor was a kind gift by Carl Thummel, PhD (University of Utah). To isolate EcR from the plasmid, PCR was performed with forward primer 5'-AATTGGTACCCAGGATGGCTATGAG-3', and reverse primer 5'-TAATTCCCGGGTCCCAGATCTCCTC-3', yielding a fragment with the EcR LBD flanked with *KpnI* and *XmaI* restriction sites on the 5' and 3' termini, respectively. After digestion with these restriction enzymes, the fragment could be ligated to the EGFP plasmid containing the desired signal sequences that was also digested with those restriction enzymes.

Mutated murine estrogen receptor was a kind gift by Dr. Michael Reth (Max Plank Institute, Germany). This receptor carries the G525R mutation that renders it 1000-fold less responsive to estradiol compared to wild-type, but does respond to 4-hydroxytamoxifen (41). The ligand-binding portion of the receptor was amplified by PCR with forward primer 5'-TAGGTACCAAGAAGAATAGCCCTGCCTTGTC-3' and reverse primer 5'-TGGATCCTGGGGCATGAAGGCGGTG-3', which introduces *KpnI*

and *BamHI* restriction enzyme sites. This fragment and the desired protein-switch vector were digested with these enzymes, and subsequently ligated together.

Protein Translocation Studies

All protein constructs were induced with ligand 24 hours after transfection. Mifepristone and dexamethasone were used as ligands for constructs with PR and GR LBD respectively. Based on our previous studies showing maximum import occurring within one hour of ligand induction (14), constructs were induced with 10nM dose of ligand for 1 hour. Pictures of live cells were taken using a fluorescence microscope after 1 hour. As controls, cells were also photographed without inducing with ligand. The constructs were constructed and tested for localization simultaneously. Depending on the localization results constructs with extreme combinations (for instance, a very strong NES and a very weak NLS) were ruled out and not constructed.

Protein Import Studies

As previously, import kinetics of protein constructs was studied by conducting time lapse experiments at three different concentrations: 1nM, 10nM and 100nM. Cells (2.5ml) were plated in live cell chambers after transfection by electroporation (8). After 18-24 hours of transfection, media was removed, cells were washed three times with PBS without calcium, and 2.5 ml of fresh media were added. Cells were incubated at 37 ^{o}C for 45 minutes prior to induction. Pictures were taken without ligand, and also with ligand induction, at time 0, 2, 5, 10, 15, 20, 30, 40, 50, 60, 90 and 120 minutes.

Quantitation of Protein Translocation

Fluorescence microscopy was used to study and quantify the amount of protein present in a cellular compartment as done previously by us and others (8, 31, 42, 43). Fluorescence intensity of a particular compartment relates to the amount of protein present in that compartment; therefore, quantitation of protein in the nucleus and cytoplasm was carried out by measuring the fluorescence intensity of EGFP, tagged to the protein construct, as previously described (8, 30). All the images were analyzed using analySIS® software (Soft Imaging System, Lakewood, CO). The nuclear and cytoplasmic intensity values were divided by the area values of the nucleus and cytoplasm to obtain average nuclear and cytoplasmic intensities, respectively, to normalize for differences in cell shape and size. Relative nuclear intensity was calculated by dividing the average nuclear intensity with average cytoplasmic intensity. Percentage nuclear increase is the increase in the nuclear intensity (which indicates the increase in the total amount of protein present in the nucleus) on ligand induction. Change in relative nuclear intensity on ligand induction, are calculated as below to compare different versions of protein constructs.

Average Nuclear Intensity = $\left(\frac{NI}{NA}\right)$ (Equation 2.1)

Average Cytoplasmic Intensity = $\left(\frac{CI}{CA}\right)$ (Equation 2.2)

Percentage Nuclear = $\left(\frac{ANI}{ANI + ACI}\right)x100\%$ (Equation 2.3)

Percentage Nuclear Increase = $\left(\frac{PN - PNo}{PNo}\right)x100\%$ (Equation 2.4)

Relative Nuclear Intensity = $\left(\frac{ANI}{ACI}\right)$ (Equation 2.5)

Relative Nuclear Intensity Change (percentage) = $\left(\frac{RNI - RNIo}{RNIo}\right)x100\%$

(Equation 2.6)

Where NI = nuclear intensity, NA = area of nucleus, CI = cytoplasmic intensity, CA = area of cytoplasm, ANI = average nuclear intensity, ACI = average cytoplasmic intensity, PN = percentage of total protein present in the nucleus 1hr. after ligand induction, PNo = percentage of total protein present in the nucleus without ligand induction, RNI = relative nuclear intensity 1hr. after ligand induction, RNIo = relative nuclear intensity without ligand induction.

Statistical Analysis

Ligand induction and import studies for all constructs were repeated in triplicate (n=3), and 10 cells representative of the whole population were analyzed in each experiment. Statistical differences between the relative nuclear intensity values were resolved using unpaired t-test with Welch's correction. One way ANOVA with Tukey's Multiple Comparison Post test was used to assess differences between RNI and PNI values for all constructs.

Results and Discussion

In our previous study (8), we established that intracellular localization of an exogenous protein can be controlled by using a protein construct containing NES, NLS and LBD. This construct is present in the cytoplasm in the absence of ligand

and translocates to the nucleus on addition of ligand. In this study we aimed to rationally design constructs which were more cytoplasmic in the absence of ligand and more nuclear in the ligand presence compared to the prototype devised in our previous study. We demonstrated that the amount of protein present in a particular compartment can be regulated by using NES and NLS of different strengths and in different combinations. It was also seen that localization controllable protein constructs can be made responsive to more than one ligand, and also show a certain degree of dose-responsiveness before saturation.

As seen in Figure 3.1, the addition of ligand caused a change in localization of the protein constructs. Most of these tested protein constructs showed a significant change ($p<0.05$) in localization from the cytoplasm to the nucleus on ligand induction. However, as noted in Figure 3.2 this change in amount of protein in the nucleus varied for each protein construct. The localization of protein depends on the strengths of NES and NLS, while the LBD provides a means to externally control this localization with a ligand. In the absence of a ligand, protein constructs are mostly cytoplasmic due to a dominant NES, while on addition of ligand, changes in conformation of LBD make the constructs translocate to the nucleus via a now dominant NLS. The amount of protein entering the nucleus depends on the critical balance between the strengths of NES and NLS. This balance of NES-NLS strengths has not been given numeric value in this study because relative strengths of some of the signals used in this study are not known. Further, the balance indicates that the protein constructs are responsive to the ligand and show a change in localization. A lack of balance would make the constructs refractory to ligand and result in localization of constructs to one particular compartment.

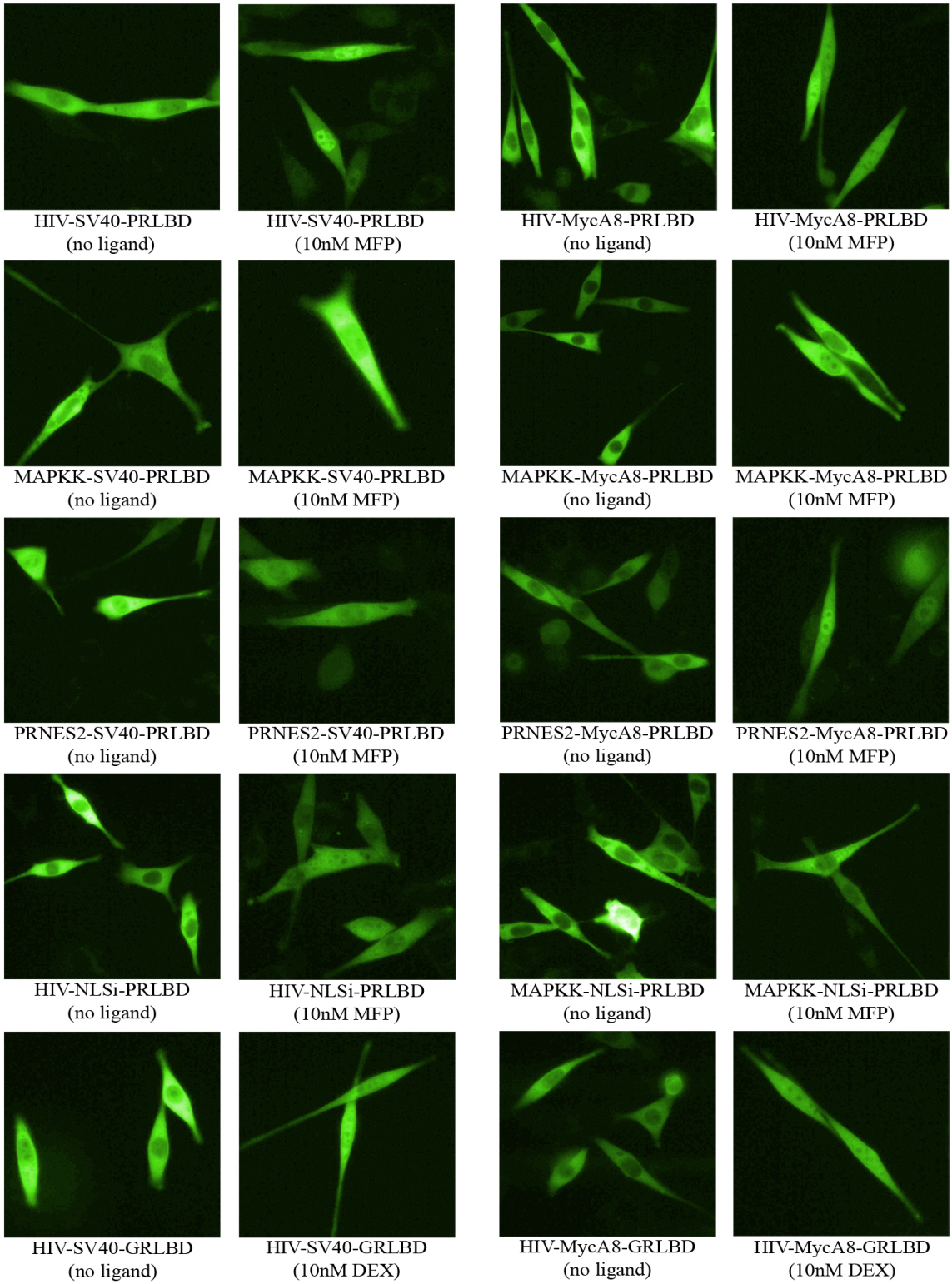

Figure 3.1. Change in protein localization on ligand induction. Change in localization of protein is observed after induction with ligand for 1hour. 10nM doses of MFP and DEX were used.

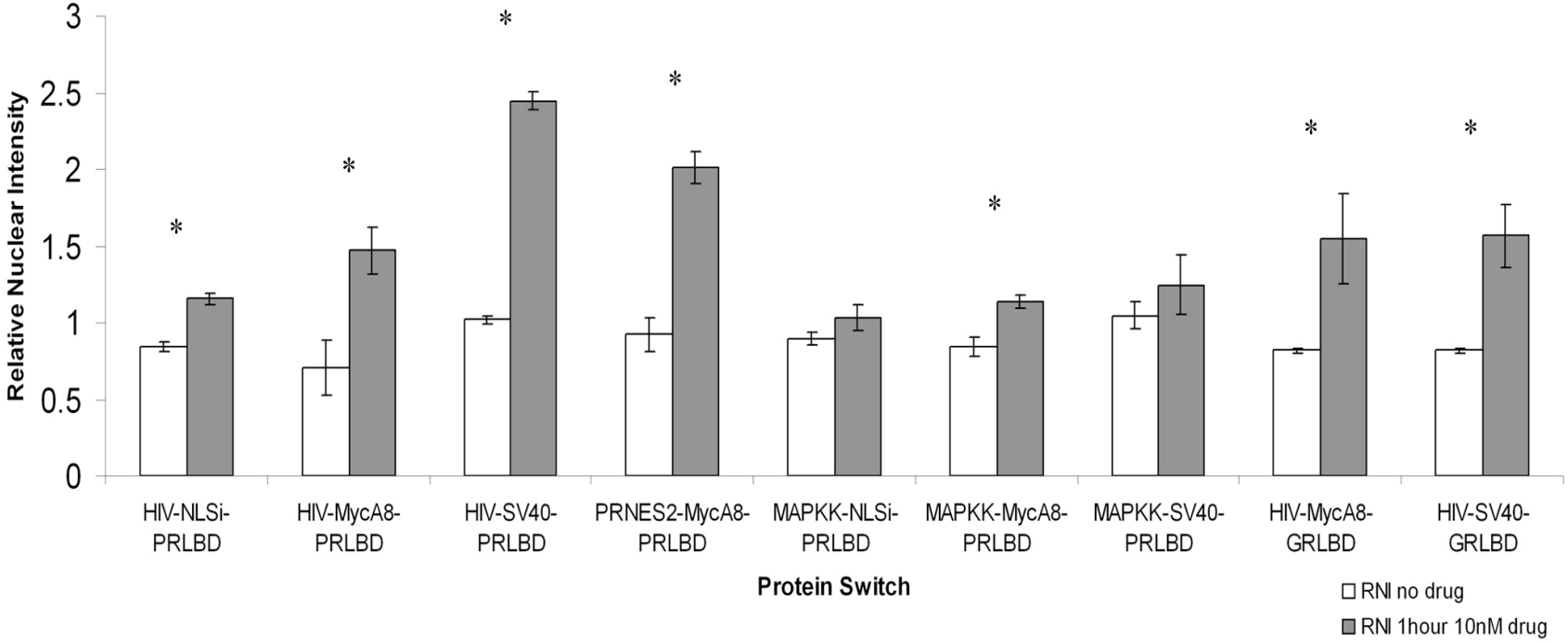

Figure 3.2. Change in relative nuclear intensity of protein constructs on induction with 10 nM ligand for 1 hour. This graph depicts the change in nuclear intensity of protein constructs on induction with 10 nM dose of ligand for 1 hour. Relative nuclear intensity for each construct was calculated by dividing average nuclear intensity with average cytoplasmic intensity. A significant difference ($p<0.05$) was observed in relative nuclear intensity values before and after ligand induction for most of the constructs. MFP was used as a ligand for constructs with PR LBD and Dex for constructs with GR LBD. (*significant difference between drug and no drug, $p<0.05$)

Changing the Strength of NLS Regulates the Amount of Protein Transported to the Nucleus

Since nuclear localization signals vary in strength, the effect of changing the NLS sequence in the presence and absence of ligand was studied (16). Starting from one of the original plasmids, HIV-NLSi-PRLBD (8), other constructs were cloned containing constant NES and LBD sequences and substituting NLSs of different strengths (one "medium" strength NLS from MycA8 (16), and a "strong" NLS from SV40 (16) and others (16, 37); see Table 3.2). Percentage nuclear increase and change in relative nuclear intensity values were calculated (as described in methods) to compare different protein constructs. As shown in Figure 3.2, HIV-SV40-PRLBD (3rd pair of columns) had the maximum relative nuclear intensity on ligand induction with 10nM mifepristone, and HIV-NLSi-PRLBD (1st pair of columns) the least. It was also observed that the difference in relative nuclear intensities in the presence and absence of ligand was greater for HIV-SV40-PRLBD than HIV-NLSi-PRLBD. The difference in relative nuclear intensity values for these constructs was presumably due to the variation in strengths of NLSs used. Studies have shown that SV40 NLS is one of strongest NLSs known, as suggested by its low Kd (dissociation) value for interaction with importin α (16). The lower the Kd value of a NLS, the stronger the binding with importin α and hence greater nuclear import (16).

Though NLSi does not have a published value for its interaction with importin α, it has been reported to have very weak import activity (35). NLSi has not been directly tested in comparison to SV40 NLS or MycA8 NLS, though. From relative nuclear intensity values in Figure 2.2, it is observed that the amount of protein present in the nucleus even without ligand induction was more for HIV-SV40-PRLBD (3rd pair of columns) than HIV-NLSi-PRLBD (1st pair of columns), due

Table 3.2

Combinations of NES, NLS, and LBD Used in this Study

NES	NLS	LBD
HIV	NLSi	PRLBD
HIV	SV40	PRLBD
HIV	MycA8	PRLBD
HIV	SV40A1	PRLBD
HIV	SV40A7	PRLBD
MAPKK	NLSi	PRLBD
MAPKK	SV40	PRLBD
MAPKK	MycA8	PRLBD
MAPKK	SV40A4	PRLBD
MAPKK	PKC	PRLBD
MAPKK	CDC	PRLBD
PRB NES2	SV40	PRLBD
PRB NES2	MycA8	PRLBD
HIV	SV40	GRLBD
HIV	MycA8	GRLBD
HIV	MycA8 x 2	PRLBD
HIV x 2	MycA8 x 2	PRLBD
MAPKK	SV40	EcRLBD
MAPKK	MycA8	mERLBD
MAPKK	NLsi	mERLBD

to the strong SV40 NLS. On the other hand, HIV-MycA8-PRLBD constructs had the least amount of protein in the nucleus in the absence of ligand, compared to the other two constructs (Figure 3.2, 2nd pair of columns), because of relatively weaker nuclear import activity of MycA8 NLS versus SV40 NLS (16). The difference between HIV-MycA8-PRLBD and HIV-NLSi-PRLBD in the absence of ligand was not statistically significant. On ligand induction, the relative nuclear intensity for HIV-MycA8-PRLBD (Figure 3.2, 2nd pair of columns) protein construct was also less than HIV-SV40-PRLBD (Figure 3.2, 3rd pair of columns) due to the lower strength of MycA8 NLS.

The nuclear intensity, and hence the amount of protein present in the nucleus on ligand induction is affected by the amount of protein present in the nucleus *before* ligand induction. Accordingly, the percentage nuclear increase (PNI, Figure 3.3A) and the change in relative nuclear intensity (RNI, Figure 3.3B), on ligand induction, were calculated to determine the best protein construct (the least amount of protein in the nucleus coupled with the highest protein translocation to the nucleus on ligand induction). It can be seen from Figure 3.3A that there was a 39% increase in amount of protein present in the nucleus on ligand induction for HIV-SV40-PRLBD (column 3) while a 44% increase for HIV-MycA8-PRLBD (column 2). The change in relative nuclear intensity values was 140% and 109% for these two constructs respectively (Figure 3.3B, column 3 and 2). The change in PNI and RNI values for HIV-NLSi-PRLBD were only 17% (Figure 3.3A, column 1) and 38% (Figure 3.3B, column 1), respectively. Both new constructs, HIV-MycA8-PRLBD (Figure 3.3A column 2) and HIV-SV40-PRLBD (Figure 3.3A column 3), showed significant improvement ($p < 0.05$) in the amount of protein present in the nucleus on ligand induction over HIV-NLSi-PRLBD (Figure 3.3A column 1); however, HIV-

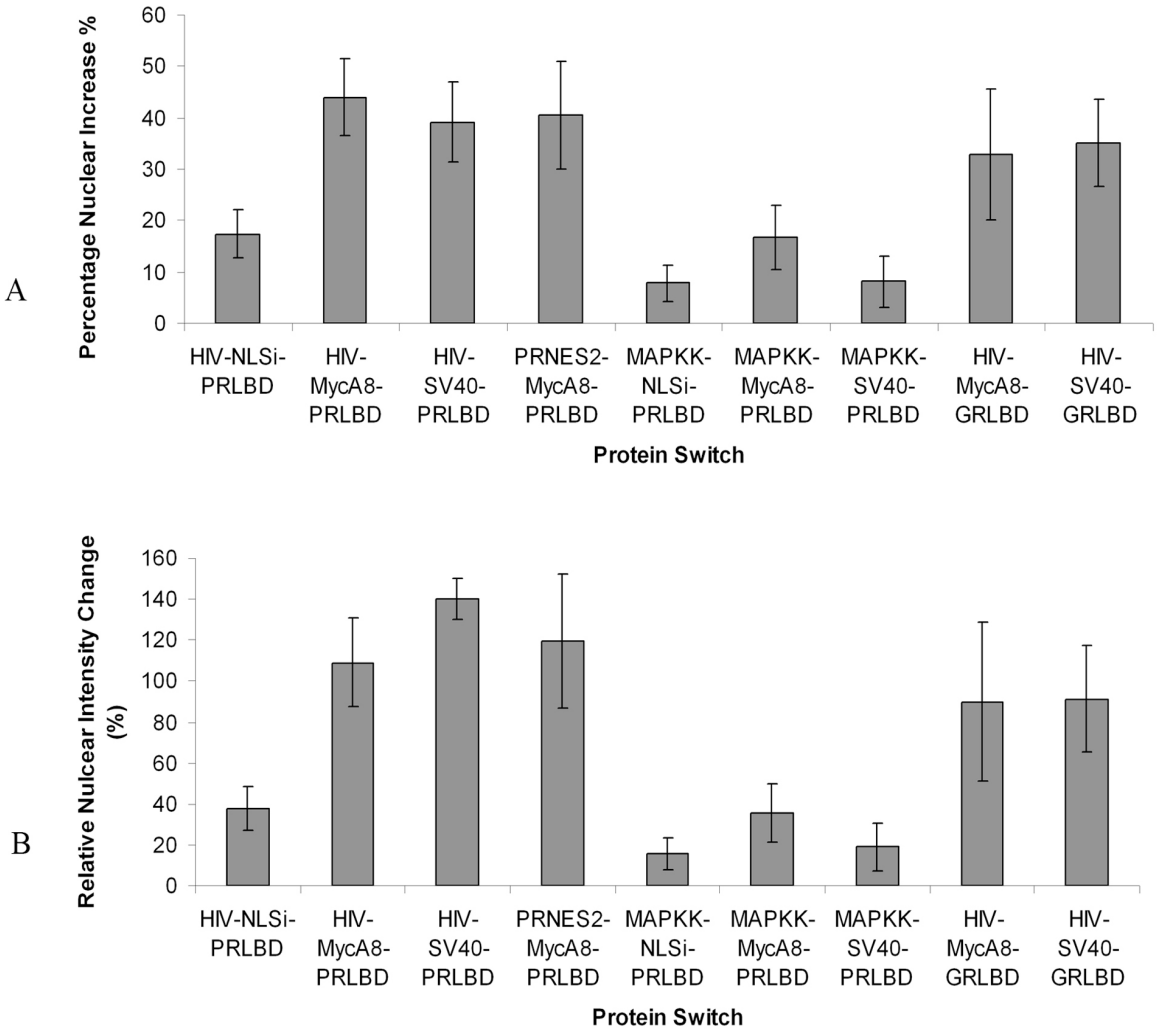

Figure 3.3. Comparison of protein constructs based on ligand induction studies. A) Percentage increase in the amount of protein in the nucleus on ligand induction for each construct is shown in this graph. The amount of protein in the nucleus was calculated in the presence and absence of ligand and the percentage increase value was calculated. A 10nM dose of MFP and DEX was used as ligand for protein constructs with PR and GR LBD, respectively. B) Change in relative nuclear intensity on ligand induction for protein constructs is depicted in this graph for different protein constructs. Relative nuclear intensity was calculated in the presence and absence of ligand, and percentage change was calculated. A 10nM dose of MFP and DEX were used as ligand for protein constructs with PR and GR LBD, respectively.

MycA8-PRLBD and HIV-SV40-PRLBD were not significantly different from each other (compare Figure 3.3A column 2 vs column 3). HIV-SV40A1-PRLBD and HIV-SV40A7-PRLBD constructs tested did not show a significant change in localization on ligand induction (data not shown). These results suggest that changing the strength of NLS in the protein construct considerably changes the amount of protein in the nucleus in the presence and absence of ligand.

Changing the Strength of the NES Affects the Localization of Protein Constructs

To investigate the affect of the NES on the localization of protein constructs, the NES in the prototype HIV-NLSi-PRLBD version was changed. In one set, HIV NES was replaced by NES from MAPKK protein to make MAPKK-NLSi-PRLBD, MAPKK-MycA8-PRLBD and MAPKK-SV40-PRLBD (8) (Table 3.2). In another set of constructs, PRNES2 was used to make PRNES2-MycA8-PRLBD and PRNES2-SV40-PRLBD (Table 3.2). As seen in Figure 3.1 (d and f) and Figure 3.2 (columns 6 and 4) only the MAPKK-MycA8-PRLBD and PRNES2-MycA8-PRLBD constructs showed a significant change ($p<0.05$) in localization of protein from the cytoplasm to nucleus on ligand induction. The change in relative nuclear intensity for MAPKK-NLSi-PRLBD, MAPKK-MycA8-PRLBD and MAPKK-SV40-PRLBD was 16%, 36% and 19%, respectively (Figure 3.3B, columns 5, 6 and 7), compared to over 100% change with the HIV NES constructs (Figure 3.3B, columns 2 and 3). The percentage nuclear increase for these MAPKK constructs was also low, at 8%, 17% and 8%, respectively (Figure 3.3A, columns 5, 6 and 7). As mentioned above, of the three constructs containing MAPKK NES, only MAPKK-MycA8-PRLBD showed a significant difference in the amount of protein in the presence and absence of ligand;

however, the magnitude of the change in PNI and RNI was low. This is observed because MAPKK is a much stronger NES than HIV, and due to its greater export strength it presumably prevents movement of protein to the nucleus from the cytoplasm on ligand induction. Other MAPKK versions of the protein construct with weaker NLSs–MAPKK-CDC-PRLBD, MAPKK-PKC-PRLBD and MAPKK-SV40A4-PRLBD–also did not show significant change in nuclear import on ligand induction (our unpublished data).

The PRNES2-MycA8-PRLBD construct showed a significant change in localization on ligand induction, except with a different extent of localization, presumably since PRNES2 is a weaker NES than MAPKK NES. There was a 40% increase in the amount of protein in the nucleus (Figure 3.3A, column 4) and a relative nuclear intensity change of 120% (Figure 3.3B, column 4) on ligand induction. There was no significant difference observed between this construct (PRNES2-MycA8-PRLBD) and the construct containing the HIV NES (HIVNES-MycA8-PRLBD) (Figure 3.3A and B, columns 4 and 2), presumably due to comparative strengths of PRNES2 and HIV NES (8). The PRNES2-SV40-PRLBD construct did not induce well and show any change in localization (our unpublished observation). Since PRNES2-SV40-PRLBD is already quite nuclear to begin with, the import machinery may already be saturated, even without ligand. Adding ligand does not cause more to go into the nucleus. In this particular construct, the balance between the NES and the NLS is tipped to the NLS in the unliganded state, so import does not occur with ligand.

Overall, these findings indicate that the strength of NES impacts the effectiveness of protein constructs by influencing the initial localization of the construct in the absence of ligand, and the overall balance of NES-NLS strengths (8).

Protein Constructs Can Be Made Responsive to Other Ligands

The ligand-binding domain provides control over the localization. Protein constructs described above contain a truncated version of PR LBD not responsive to endogenous PR, but responsive to antagonist mifepristone (8, 44). To confer responsiveness to a different ligand, protein constructs were created using the glucocorticoid receptor LBD (C656G GRLBD). These constructs were designed to be responsive to dexamethasone (dex) (45). GR LBD with a point mutation C656G was used in these constructs because this mutated GR LBD is 9-fold more sensitive to dex (39, 45). The benefit of this particular system is that low doses of dex can be used to cause nuclear induction, and these sub-nanomolar levels of dex will not activate endogenous GRs (39). Constructs containing C656G GR LBD, HIV-MycA8-GRLBD and HIV-SV40-GRLBD, showed change in localization on ligand induction similar to constructs containing PR LBD (Figure 3.1). Upon inducing the constructs with 10nM dexamethasone, as observed in Figure 3.2, pairs of columns 8 and 9, there was a significant difference ($p<0.05$) in relative nuclear intensity values compared to uninduced. The increase in amount of protein present in the nucleus was 33% for HIV-MycA8-GRLBD constructs (Figure 3.3A, column 8) and 35% for HIV-SV40-GRLBD constructs (Figure 3.3A, column 9). The change in relative nuclear intensity values was 90% and 91%, respectively, for these two constructs (Figure 3.3B, columns 8 and 9).

A mutated version of the murine ERLBD was also examined for efficient nuclear translocation in the context of the protein switch. The G525R mutation renders the receptor unresponsive to endogenous estradiol agonism, but allows hydroxytamoxifen to agonize the receptor (41). Like the GRLBD that is hyper-

responsive to external ligand dexamethasone, a protein switch that only responds to external ligand hydroxytamoxifen (OHT) is more ideal as a potential treatment modality than one that can be activated unnecessarily by circulating hormones. The translocation results of MAPKK-MycA8-ERLBD construct can be seen in Figure 3.4A. At 2 hours, there was no visible change in nuclear localization upon induction by 100 nM OHT. To rule out the possibility that a higher dose, or a greater time exposure time was needed for this particular LBD, MAPKK-MycA8-ERLBD was then exposed to 1000 nM OHT for 24 hours. Again, no change in localization was be seen under these conditions, suggesting that either the LBD does not produce a conformational change needed to expose the NLS, or the particular MAPKK-MycA8 combination is not effective. The MAPKK-NLSi-ERLBD combination also resulted in no visible nuclear localization under the two treatment conditions.

The opportunity to use a completely non-native ligand-binding domain that would not respond to any endogenous hormones can be found in the insect EcR. This receptor is also part of the nuclear receptor superfamily, but is only found in insects. Its main purpose is to control insect molting transitions, and its ligands are thought to be non-toxic to humans (40, 46). The EcR LBD was fused to MAPKK-MycA8 and tested for ligand responsiveness of insect ecdysteroid muristerone A (Figure 3.4B). A dose of 100 nM was tested at 1 hour, 2 hours, and 4 hours with apparently similar results at these times. These results also suggest that either the combinations of NES and NLS were not optimal, or that the conformational change of EcR LBD in response to ligand is not sufficient to produce a translocation response of the protein switch. A quantitative measurement of nuclear protein after drug addition is represented in Figure 3.4C, which shows MAPKK-MycA8-EcRLBD having 4.4±1.9 %

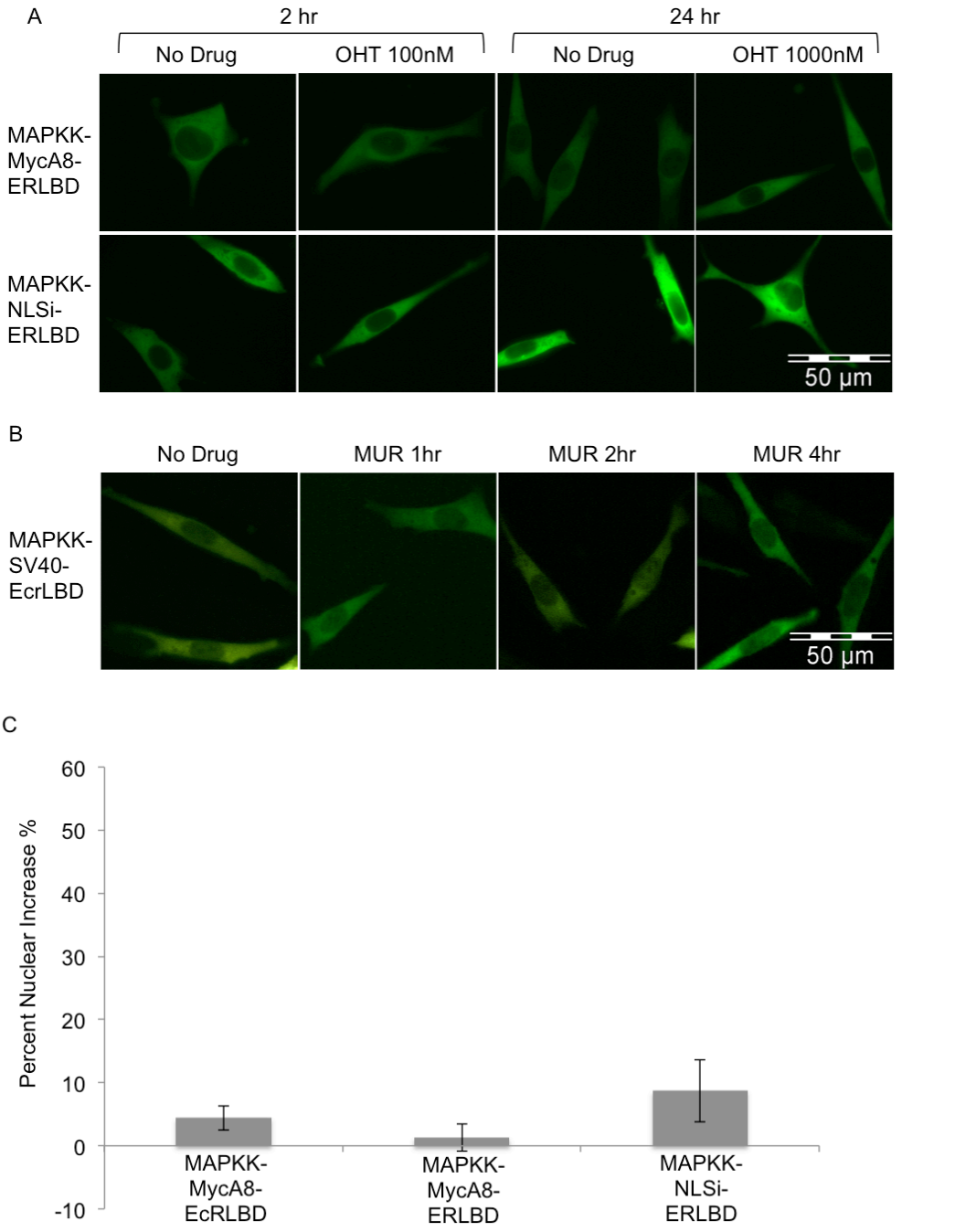

Figure 3.4. Additional ligand-binding domains for inducible nuclear localization. A) The mouse ER LBD was fused to MAPKK-MycA8 (top row) and MAPKK-NLSi (bottom row). Images were captured at 2 hours and 24 hours, with little visible change in localization. B) The EcR LBD does not demonstrate a visible nuclear localization change after addition of 100 nM muristerone A (MUR) after 1, 2, or 4 hours.C) Percent nuclear increase of constructs after 2 hours of respective ligand addition. Plotted on the same scale as Figure 3.3 A.

(mean±SEM), MAPKK-MycA8-ERLBD having 1.3±2.2 %, and MAPKK-NLSi-ERLBD having 8.7±4.9 % nuclear increase in response to their respective ligands. These results were plotted on the same scale as other other protein switch combinations (Figure 3.3A). These results were comparable to those seen with other MAPKK-MycA8 and MAPKK-NLSi constructs with PRLBD. Taken together, it is most likely that the combination of signal sequences was suboptimal, and the contribution of these LBDs is inconclusive.

To conclude, protein constructs for controlled intracellular localization of proteins can be customized with different ligand binding domains to confer responsiveness to a range of ligands. Responsiveness of protein switch to different ligands will provide greater flexibility and ease of therapy by utilizing already available and tested noncontroversial and nontoxic ligands.

Multiple NLS and NES Render the Protein Construct Unresponsive to External Ligand

In order to study the effect of multiple localization signals on nuclear import of protein constructs a second MycA8 NLS was added to HIV-MycA8-PRLBD, resulting in HIV-MycA8-MycA8-PRLBD. It was observed that this construct with two NLSs was mostly nuclear even in the absence of ligand, and localization was not altered by addition of ligand (Figure 3.5). Addition of another export signal (HIV-HIV-MycA8-MycA8-PRLBD) also did not alter the nuclear localization of the constructs. No significant difference was observed in the amount of protein present in the nucleus in the presence and absence of ligand for these constructs with multiple localization signals.

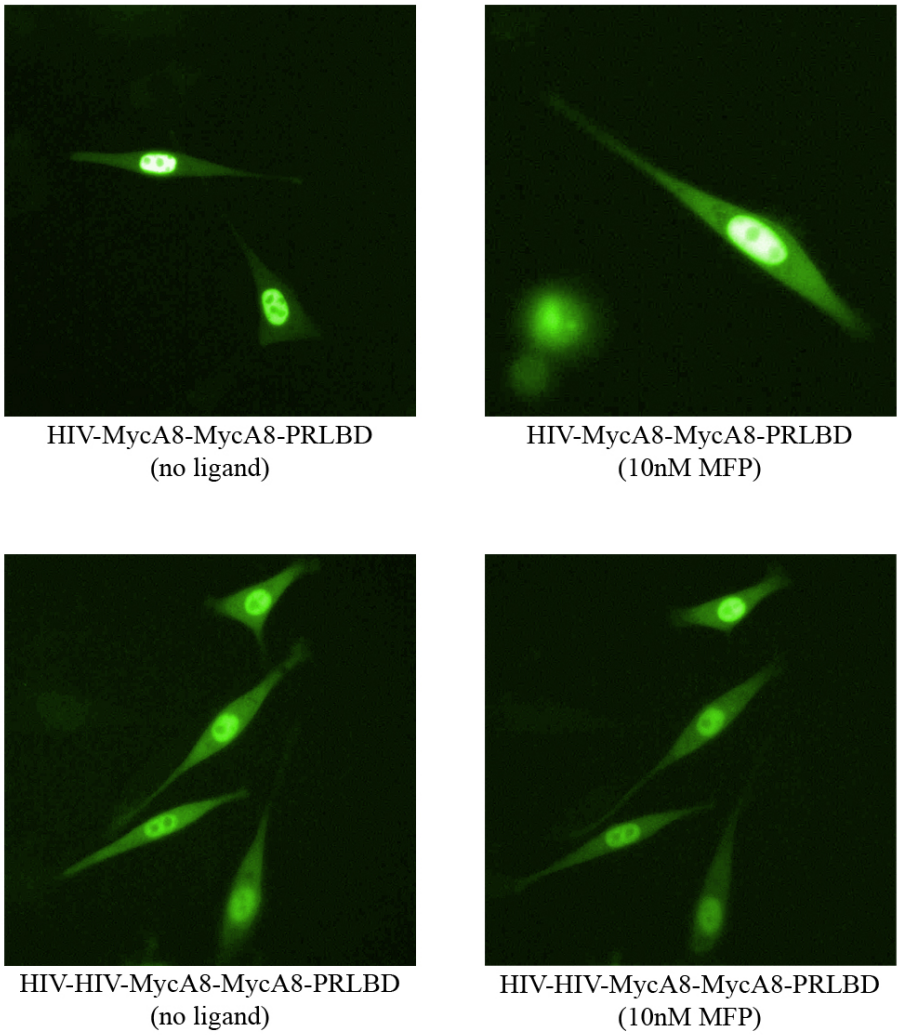

Figure 3.5. Effect of multiple signal sequences on construct localization. Addition of two NLSs made the constructs completely nuclear even in the absence of ligand. No change in localization was observed on adding a second NES to these constructs. Significant difference was not observed in localization of these constructs in the presence and absence of ligand.

To summarize, various protein constructs were made by using classical NESs—HIV, MAPKK and PRNES2—in combination with SV40 T-antigen type NLSs of various strengths (Table 3.2). Most of the constructs tested showed a significant change in localization on ligand induction. All of the new constructs reported in this study showed a significant improvement in control of localization over our previous prototype constructs. New constructs containing stronger NLSs than the one used in our previous study (NLSi) showed a much greater increase in the amount of protein in the nucleus on ligand induction, signifying that the strength of NLS used influences the overall translocation of the construct, shifting the localization of constructs to the nucleus. It was seen that stronger NLSs, due to their greater interaction with import receptors (16), tend to shift the protein to the nucleus even in the absence of ligand. Similarly, the strength of NES also influences the localization of protein constructs, however, in the opposite direction. In all the constructs used in this study containing MAPKK NES, addition of ligand did not cause a significant change in localization from the cytoplasm to the nucleus. This indicated that a strong NES, such as MAPKK, tends to keep the constructs mostly in the cytoplasm in the absence of ligand. We also observed that addition of multiple repeats of localization signals tends to shift the balance of localization towards one compartment to a greater degree, making it unresponsive to the ligand. The construct containing “medium” strength HIV NES, “medium” strength MycA8 NLS and PR LBD showed most favorable results with the least amount of protein present in the nucleus in the absence of ligand, and the greatest translocation to the nucleus with ligand. Hence, ultimately it is the balance of NES and NLS strengths that determines the overall localization of protein constructs in the presence and absence of ligand. This balance can be manipulated to control the amount of protein

translocation to a compartment. It was also observed that the rate of nuclear import of the protein constructs depends on the dose of the ligand used, as well as the inherent import characteristics of the LBD used. These protein constructs can however be made responsive to more than one ligand. In our study, we successfully tested two sets of constructs containing truncated PR LBD and mutated C656G GR LBD, responsive to mifipristone and dexamethasone, respectively. The ability to substitute LBDs to make localization controllable protein constructs provides the advantage of using various nontoxic, previously tested ligands, thus allowing creation of customized protein constructs.

Intracellular transport and localization of proteins is significant for normal functioning of cells, and any deviation from this highly regulated transport system results in diseases which may range from metabolic disorder to cancer (3, 7, 47-50). The constructs described here exploit nuclear localization and nuclear export signals to optimize a system for controlling intracellular localization of proteins. Control over intracellular protein localization may render a protein inactive in one compartment and active in another; thus manipulating the localization of a protein using an external ligand would provide more control over the therapy. An endogenous gene, with native localization signals intact, may still not localize to its correct compartment in the cell on transfection, due to malfunctioning of transport, mutations or other cellular events that are difficult to control (8). However, attaching the gene with localization controlled protein constructs would render the ability to target the gene (and hence protein) to its desired compartment with greater control and assurance. Changing the localization of a protein by utilizing signal sequences to treat diseases is advancement over current gene therapy. Many diseases are caused due to mislocalization of proteins. Various types of cancers,

ranging from breast cancer to certain leukemia's involve mislocalization of proteins (51). Most of these cancers involve mislocalization between the nucleus and the cytoplasm. In our recent review we stressed the need of exploring modes for controlling intracellular localization of proteins (51). Localization controllable protein constructs with a range of NES-NLS combinations studied in this paper is one such approach, exploiting intracellular localization for treatment of diseases. A protein which is mislocalized in a disease state can be directed to its "normal" compartment by using localization controllable protein constructs. These constructs can also be used in states where there is inadequate production of a certain protein by using a construct with appropriate NES-NLS balance to regulate the amount of protein translocating to a compartment.

Conclusions

The optimization of localization controllable protein constructs was carried out in this study. Classical NESs from HIV-rev, PR and MAPKK proteins were used in combination with SV40 T-antigen NLSs of various strengths, along with a LBD to make the constructs. The overall localization of the constructs was influenced by the individual strengths of the localization signals used, as well as the critical balance between the NES-NLS strengths. A strong NES, such as MAPKK, tends to keep the constructs cytoplasmic and did not show any significant change in localization with ligand. On the other hand, a strong localization signal such as SV40 NLS, caused translocation of the protein constructs to the nucleus even in the absence of ligand. Using multiple repeats of localization signals also tend to shift localization of the constructs towards one compartment. Thus, the NES-NLS used in a protein construct can be manipulated to regulate the amount of protein translocating to a

compartment. In this study, protein constructs were made by using two different LBDs responsive to their respective ligand providing a means of making customized constructs. The optimized versions of localization controllable constructs also showed a significant change in import rate at various ligand doses. It was observed that the change in rate of nuclear import was affected by the type of LBD used. The optimized localization controllable protein constructs discussed in this study provide a mean for regulating the amount of protein present in a compartment, and protein translocation across various intracellular compartments, and pave the way for treating diseases which are caused due to mislocalization of proteins.

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CHAPTER 4

UTILIZING THE ESTROGEN RECEPTOR LIGAND-BINDING DOMAIN FOR CONTROLLED PROTEIN TRANSLOCATION TO THE INSOLUBLE FRACTION

Abstract

The estrogen receptor forms insoluble aggregates in the cytoskeletal subcellular fraction when bound to the antagonist fulvestrant. The ligand-binding domain was isolated and fused to signal sequences to target subcellular compartments. Sequestering a pro-apoptotic peptide tested the utility of a protein targeted to the insoluble fraction. The ligand-binding domain of the estrogen receptor was isolated and fused with signal sequences, either a nuclear localization signal or nuclear export signal. The subcellular localization when bound to drug fulvestrant was examined, specifically its interaction with cytokeratins 8 and 18. The ability to target a therapeutic peptide to the insoluble fraction was tested by fusing a therapeutic coiled-coil from Bcr-Abl in

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Davis, J.R., Mossalam, M., Lim, C.S.
JRD developed the concept, created constructs, performed microscopy and image analysis. MM contributed with the K562 experiments and apoptotic assays. CSL contributed intellectually throughout.

K562 cells. The estrogen receptor ligand-binding domain responds to fulvestrant by translocating to the insoluble fraction. Adding a signal sequence significantly limited the translocation to either the nucleus or cytoplasm. The cytokeratin 8/18 status of the cell did not alter this response. The therapeutic coiled-coil fused to ERLBD was inactivated upon ligand induction. Isolating the ligand-binding domain of the estrogen receptor creates a ligand-controllable protein capable of translocation to the insoluble fraction. This can be used to sequester an active peptide to alter its function.

Introduction

In nature, many proteins function exclusively in one subcellular compartment or another. Therefore, tight regulation of protein subcellular location provides a way for cells to control protein function in the complex cellular milieu. Much work in the past decade has focused on discovering, defining, and utilizing signal sequences for regulating protein location in cells (1, 2). Subcloning allows the addition or removal of signal sequences to known proteins for altered location and function (1, 3, 4). For example, we have shown that it is now possible to send the normally oncogenic protein Bcr-Abl, the causative agent in chronic myelogenous leukemia, to the nucleus, where it instead acts as an apoptotic factor (5). We also have shown that targeting p53 (a tumor suppressor) or c-Abl (a proto-oncogene) to the mitochondria can cause apoptosis (6, 7). Our laboratory has also pioneered the concept of the "protein switch" where the location of a cytoplasmic protein can be made nuclear in a ligand-dependent fashion (3, 4). We were also interested in creating different kinds of protein switches that would be capable of capturing active proteins and sequestering them into other subcellular compartments, rendering

them inactive. This paper, to our knowledge, is the first to describe a protein switch capable of translocating from the nucleus or cytoplasm (or both) to an insoluble cytoskeletal fraction. This protein switch utilizes a version of the estrogen receptor ligand-binding domain (ERLBD) that is exclusively responsive to fulvestrant, a non-natural ligand that can be used for exquisite regulation of the protein location in the cell. We provide proof of concept of dysregulation of an active peptide (8) whose function is altered when triggered to the cytoskeletal fraction upon addition of ligand.

The ERLBD is derived from the estrogen receptor (ER), a member of the steroid hormone nuclear receptor (SHR) family (9). SHRs have an N-terminal activation domain, a central DNA binding domain, and a C-terminal ligand binding domain (LBD) (10). As with other steroid receptors, ER functions as a transcription factor when bound to its cognate agonist by dissociating from chaperone proteins and binding target DNA or other proteins involved in gene transcription (11, 12). The stability of the ER has been shown to be dependent upon the ligand to which it is bound (13). In addition to activating gene transcription through the activation function domains, the endogenous ligand estradiol (E2) destabilizes ER levels by increasing proteasomal degradation and receptor turnover. The drug tamoxifen (one of the members of the selective estrogen receptor modulators, or SERMs) is a partial agonist at the ER, but also acts to stabilize the receptor. Fulvestrant (ICI 182,780, Faslodex®) is a member of the selective estrogen receptor down-regulators (SERDs) which act to disrupt the typical nucleocytoplasmic shuttling of ERα and result in cytoplasmic accumulation in a protein-synthesis dependent process (14, 15). Fulvestrant is a clinically useful antiestrogen that prevents transcriptional

activation and rapidly degrades ERα (13, 14, 16-22). The bulky side chain of fulvestrant induces a conformational change of helix 12 within ERα's LBD that increases hydrophobicity (23, 24). Lupien *et al.* suggest receptor insolubility follows antagonist binding, mediated by hydrophobic residues L356, L540, and M543 residing in the helix 12 of the LBD (25). In HepG2 cells, the disappearance of the estrogen receptor after fulvestrant treatment appears to be caused by failure to extract the insoluble ER aggregates, though in MCF-7 cells receptor insolubility and degradation was observed. Long *et al.* speculate that the degradation of ER in the presence of fulvestrant is due to cytokeratins 8 and 18, which facilitate proteasomal degradation of ERα (19). Proteasomal degradation of ERα requires a functional helix 12 (19), and an active transcriptional activation complex (26-28). Removal of activation domains also prevents transactivation in the presence of receptor agonists, such as 17ß-estradiol. Cytokeratins 8 and 18 are the primary intermediate filaments of single-layer epithelial cells, and play a variety of roles including cell-cycle regulation, protection from cell stress and apoptosis (see Moll *et al.* and references within) (29). Unsurprisingly, these filaments may be useful in diagnostic immunohistochemistry, and may also be involved in the oncogensis of cutaneous squamous cell carcinoma (30), and may confer a favorable prognosis in breast carcinomas, likely due to the facilitation of ERα degradation in ER+ tumors (19, 31, 32).

A GFP fusion protein with the ligand-binding domain of ER (ERLBD) was utilized to target the insoluble cytoskeletal fraction when bound to fulvestrant. The role of cytokeratins 8 and 18 in ERLBD cytoskeletal targeting is also examined. We show that fusing either a nuclear localization signal (NLS) or nuclear export signal

(NES) to ERLBD can confer subcellular compartment specificity, thus allowing the ERLBD cytoskeleton interaction to occur either in the cytoplasm or nucleus. We also present an example of a protein capable of being sequestered in the insoluble fraction via the ERLBD, rendering it inactive.

Materials and Methods

Cell Culture

The murine breast adenocarcinoma cell line 1471.1 (a kind gift from G. Hager, NCI, NIH) and human ovarian carcinoma cell line A2780 (Sigma Aldrich, St. Louis, MO) were grown as monolayers in DMEM (Invitrogen, Grand Island, NY) supplemented with 10% FBS (Invitrogen), 1% penicillin-streptomycin-L-glutamine (Invitrogen), 0.1% gentamicin (Invitrogen). Human breast adenocarcinoma MCF-7 cells (ATCC, Manassas, VA) were grown in monolayer in RPMI media (Invitrogen) supplemented as above, with the addition of insulin 4 mg/L (Sigma Aldrich). Monkey kidney fibroblast COS-7 cells were cultured as monolayer in RPMI media with antibiotic supplements. All cells were maintained in 5% CO_2 incubator at 37ºC.

Plasmid Construction

A plasmid containing the ligand-binding domain of the human estrogen receptor α (amino acids 302 to 553, ERLBD) was a kind gift from Carolyn Smith (Baylor College of Medicine). To subclone ERLBD into the multiple cloning site (MCS) of EGFP-C1, a KpnI restriction site was introduced before the ERLBD by site-directed mutagenesis with top primer 5'-GGACAAGGCCAGGCTGTT-CTTCTTAGAGGTACCAC-CGGATCTAGATAACTGATC-3' and bottom primer 5'-GATCAGTTATCTAGATCCGGTGGTACCTCTAAGAAGAACAGCCTGGCCTTGTCC

-3'. The KpnI digested ERLBD fragment was subcloned into the KpnI site of EGFP-C1 resulting in EGFP-ERLBD. Sequence analysis (at the University of Utah Core DNA Sequencing Facility) verified successful cloning.

To create EGFP-NLS-ERLBD, the NLS sequence from SV40 large T-antigen was placed between BspEI and XhoI sites. The oligonucleotides containing the NLS sequence were synthesized by the core facility, and were annealed together, creating a double stranded insert flanked by BspEI and XhoI overhangs. The sequences for the oligonucleotides are as follows: top strand: 5'-CCGGAAGCCCG-AAAAAAAAACGCAAAGTGGAATC-3' and bottom strand: 5' TCGAGATTCCAC-TTTGCGTTTTTTTTTCGGGCTT-3'. The annealed oligonucleotides were ligated into a digested EGFP-ERLBD vector. Similarly, the NES from mitogen activated protein kinase kinase (MAPKK) was cloned between BspE1 and XhoI to form EGFP-NES-ERLBD. The oligos were synthesized as follows: top strand: 5'-CCGGACTGCAGAAAAAACTGGAAGAACTGGAACTGTC-3' and bottom strand: 5'-TCGAGACAGTTCCAGTTCTTCCAGTTTTTTCTGCAGT-3'. In addition, the EGFP-ERLBD-CCmut3 was constructed by amplifying the DNA encoding ERLBD by polymerase chain reaction, using the primers 5′-GGATCACTCTCGGCATGG-3′ and 5′-GCGCGCGCGCTCCGGAGCTAGTGGGCGCATGTAGG-3′ followed with BspEI digestion. This was subcloned into the BspEI restriction enzyme site in our published EGFP-CCmut3 plasmid (8).

Transient Transfections

For microscopy studies, cells were plated on a clear cover glass in six well plates (Greiner Bio-One Cellstar, Monroe, NC) or live cell chambers (Nalg Nunc, Rochester, NY) with corresponding media the day before transfection. For western

blot, cells were plated in 60 mm dishes (Corning Inc., Corning, NY). Transfections were performed with Lipofectamine 2000 (Invitrogen) per manufacturer's recommendations. K562 cells were transfected according to the Cell Line Nucleofector Kit V Protocol (program T-013) using the Amaxa Nucleofector II (Lonza Group, Basel, Switzerland).

Microscopy and Image Analysis

Approximately 16 to 24 hours after transfection, cells were viewed on an Olympus IX701F inverted fluorescence microscope (Scientific Instrument Company, Aurora, CO) as previously described (33). To quantitate protein location, the fluorescent images obtained were analyzed using analySIS® software (Soft Imaging System, Lakewood, CO) as previously described (4). To obtain average cytoplasmic intensity data for all constructs, 10 representative cells from each experiment were analyzed and averaged from 3 separate experiments (n=3), and are expressed as percent cytoplasmic intensity. Statistical differences between the constructs were determined using one-way ANOVA with Tukey's post-hoc test.

Western Blot

Approximately 24 hours after transfection, cells were treated as indicated, briefly rinsed with PBS, removed from the dish by trypsinization, then centrifuged at 500 x *g* for 5 minutes. Cell lysis and subcellular fractionation were carried out with the Subcellular Protein Fractionation Kit (Thermo Scientific). Cytoplasmic and cytoskeletal fractions were separated on a Novex Bis-Tris 10% gel (Invitrogen), transferred to PVDF membrane by iBlot (Invitrogen). Blocking was accomplished

with Blocker BLOTTO (Thermo Scientific, Rockford, IL). Primary antibodies were diluted in Blocker BSA 2% (Thermo Scientific) in TBST. For detection of cytokeratin 18, a rabbit polyclonal to cytokeratin 18 (Abcam ab52948, Cambridge, MA) was used, diluted to 1:10,000. For detection of GFP, rabbit polyclonal Anti-GFP (Sigma Aldrich G1544) was diluted to 1 µl/mL. Loading control anti-GAPDH (Cell Signaling #5174, Danvers, MA) was diluted to 1:1000. Secondary anti-rabbit IgG conjugated to horseradish peroxidase (Cell Signaling #7074) was diluted 1:2500 in BSA 2% in TBST. To produce chemiluminescence, the SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific) was incubated on the membrane. Western images were acquired with FluorChem FC2 imaging station with AlphaView Software (AlphaInnotech, San Leandro, CA).

Indirect Immunofluorescence

Approximately 24 hours after transfection, MCF-7 cells were treated with drug or vehicle for 1 hour, and then rinsed briefly with ice-cold PBS. Cells were fixed with 4% paraformaldehyde solution for 15 minutes at room temperature, and then washed twice with ice-cold PBS. Cell membranes were permeabilized with 0.5% Triton X-100 in PBS for 10 minutes, and then washed with PBS three times for 5 minutes each. Blocking was done with BLOTTO for 1 hour. Primary antibody against CK18 was diluted 1:500 in 5% BSA in PBS, and then incubated with cells for 1 hour at room temperature. Cells were washed x 3 with PBS for 5 minutes. Goat anti-rabbit secondary antibody conjugated to Texas Red (Abcam ab6719) was diluted 1:500 in BLOTTO and incubated with cells for 1 hour at room temperature. Excess antibody was removed by washing x 3 for five minutes. Cover slips were mounted to glass slides with Fluoromount G (Electron Microscopy Sciences, Hatfield, PA). Fixed

slides were viewed with an Olympus IX81 FV1000-XY confocal microscope (Imaging Core Facility, University of Utah) with a 60X objective and 3.0x digital zoom as previously described (8). Excitation and emission filters for EGFP and Texas Red were 488 nm excitation, 500-530 nm emission filter, and 543 excitation, 555-655 nm emission filter, respectively. To account for variations in expression of EGFP, the exposure settings and laser gain of each channel were kept below detected pixel saturation for each cell. Olympus FluoView software was used to capture images; ImageJ (http://rsb.info.nih.gov/ij) was used for image analysis. Prior to statistical analysis, cell images were corrected for background noise. Identical regions of interest (ROIs) were created around whole cells for each fluorophore, and these ROIs were compared using JACoP colocalization plugin to estimate Costes' colocalization coefficient (34, 35). To better visualize colocalization, the Colocalization ColorMap plugin was used (36).

7AAD Assay

K562 cells were stained with 7-aminoactinomycin D (7-AAD, Invitrogen) according to manufacturer's instructions 48 and 72 hours after transfection. The samples were analyzed using the FACSCanto-II (BD-BioSciences, University of Utah Core Facility) with FACSDiva software. Only transfected cells were analyzed by gating for EGFP positive cells. EGFP and 7-AAD were excited with the 488 nm laser, and were detected at 507 nm and 660 nm, respectively. Each construct was assayed three times (n=3). The means of each group were compared by ANOVA with Tukey's post-hoc test.

Results

Subcellular Localization

Three protein constructs were constructed and transiently transfected into various cell lines. Figure 4.1 shows the results from microscopy of these cells. The first construct tested was EGFP-ERLBD, a truncation of the human ERα consisting of amino-acids 302-595. This region of the receptor is responsible for ligand binding and transactivation, but lacks the domains capable of interacting with DNA. The result is a construct that takes on whole-cell localization when viewed by fluorescence microscopy in the presence of ethanol (vehicle), but in the presence of fulvestrant takes on a punctate staining pattern. The basal localization is in contrast to full-length receptor that localizes exclusively in the nucleus, and only appears in the cytoplasm after fulvestrant addition (14). This distribution was visualized in murine breast adenocarcinoma cell line 1471.1, human breast adenocarcinoma MCF-7 cells, human cervical carcinoma HeLa cells, and monkey kidney fibroblast COS-7 cells.

To test the ability to control the subcellular localization of the construct in these cell lines, we then fused a nuclear export signal from the mitogen activating protein kinase kinase (MAPKK) "NES-ERLBD", or a classical monopartite nuclear localization signal from the SV40 large-T antigen "NLS-ERLBD" (Figure 4.2). The results demonstrate an ability to predetermine the subcellular compartment of an introduced protein. The NES excludes the introduced protein from the nucleus (Figure 3.2, left half), with or without the presence of fulvestrant, and the NLS robustly directs the protein into the nucleus (Figure 4.2, right half).

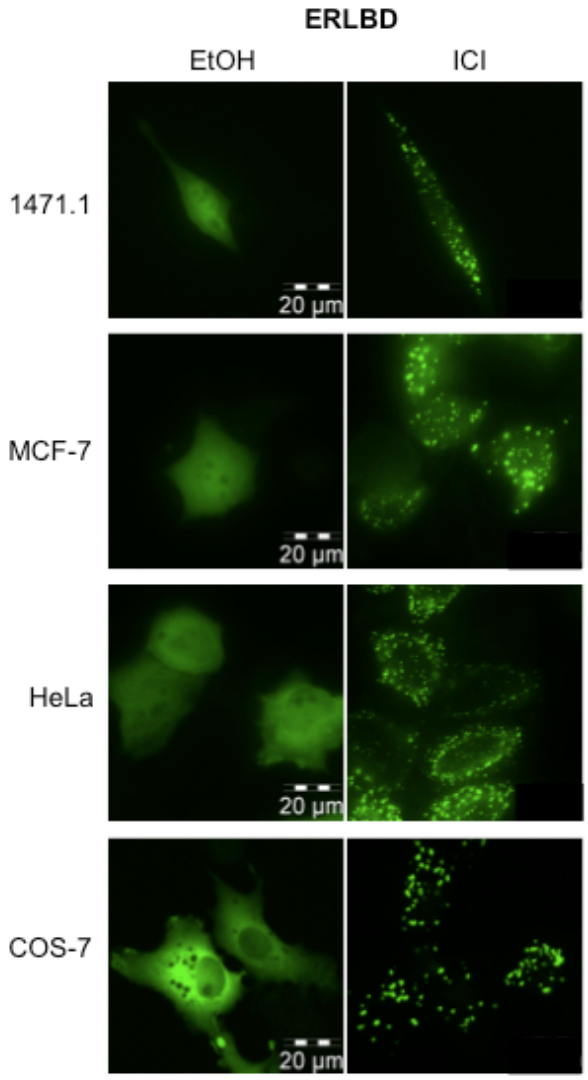

Figure 4.1. Fluorescence microscopy of cells transfected with EGFP-ERLBD. Presented here are representative fluorescence microscopy images of cells from multiple cell types (1471.1, MCF-7, HeLa, or COS-7 cells). Cells were treated with EtOH (ethanol, left) or fulvestrant (ICI, right) 100 nM for 1 hour. With EtOH treatment, the protein can be seen as having a whole-cell distribution. When treated with fulvestrant, the protein was seen in dense punctate clusters, in both the cytoplasm and nucleus.

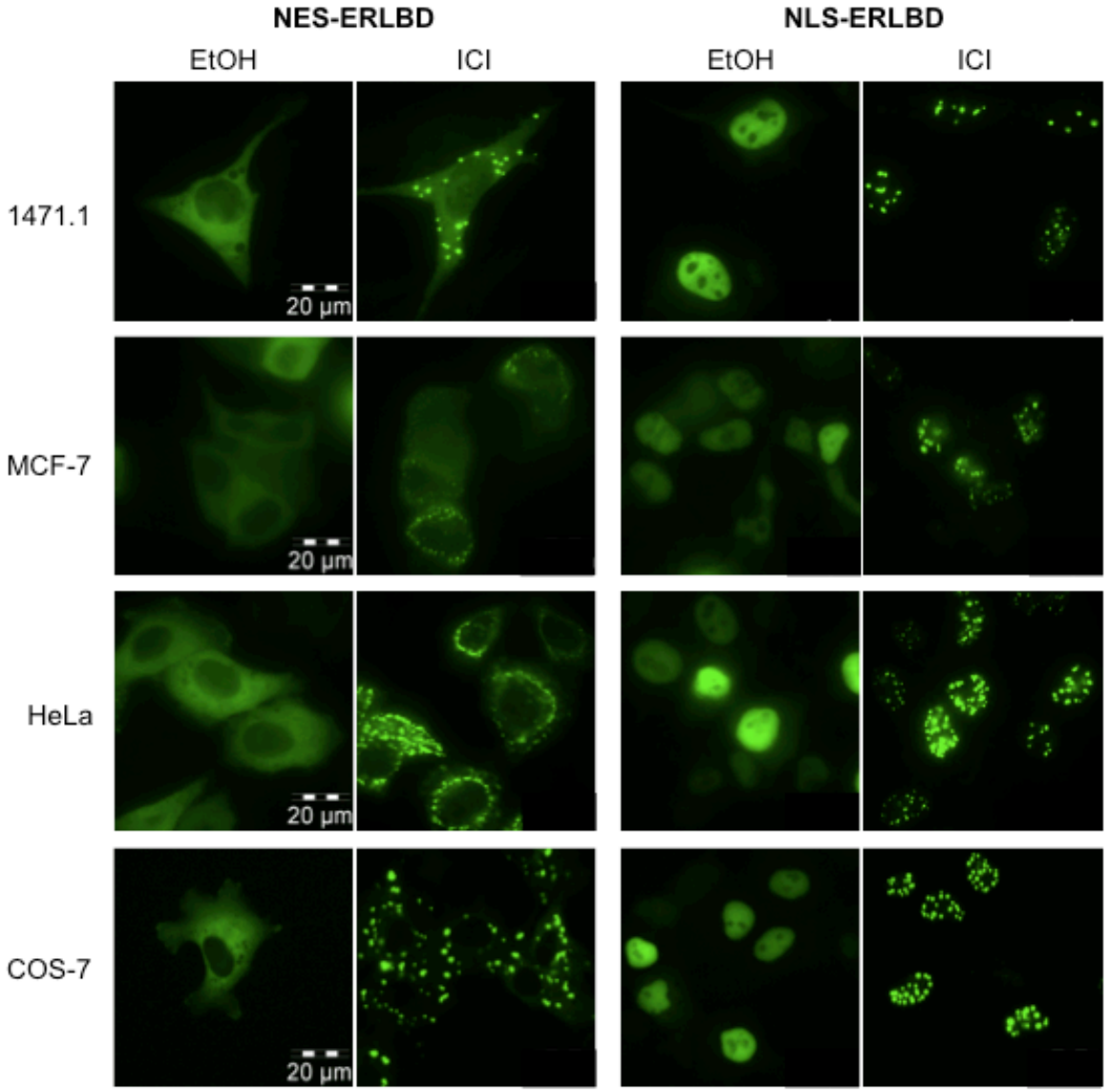

Figure 4.2. Effect of signal sequences on localization. The inclusion of signals sequences changes the distribution of ERLBD across multiple cell types (1471.1, MCF-7, HeLa, or COS-7 cells). A nuclear export signal fused to EGFP-ERLBD (left) shows a more prominent cytoplasmic distribution with or without treatment with fulvestrant 100 nM for 1 hour. A nuclear localization signal (right) clearly demonstrates a pronounced nuclear accumulation with or without fulvestrant. In both cases, a change to punctate clusters occurs in the presence of fulvestrant in their respective subcellular compartment (nucleus or cytoplasm).

Quantification of the amount of fluorescence of each construct in the cytoplasm in 1471.1 cells was done (Figure 4.3A). ERLBD was found to have 36.7% ± 2.2 (mean ± SD) cytoplasmic fluorescence, NES-ERLBD had 54.5% ± 1.5, and NLS-ERLBD had 20.9% ± 2.7. Compared to ERLBD, NES and NLS constructs were significantly different, with p-values < 0.001 by ANOVA with Tukey's post-hoc test. Figure 3B shows the fluorescence from a region within the cell before and after drug addition, demonstrating the displacement of proteins to aggregates. Figure 4.3C shows the change, in percent, of this movement. The negative value signifies the loss of fluorescence in a defined region of interest as the protein translocates away from the region to insoluble punctate dots. Only ERLBD and NLS-ERLBD showed high protein displacement after fulvestrant addition (> 80%). Nevertheless, NES-ERLBD does show a change in the cytoplasmic pattern, going from a dispersed to a punctate pattern, but no overall displacement of fluorescence intensity in that region.

ERLBD Localization by Fractionation

It has been proposed that the distribution change of ER when bound to fulvestrant is due to an interaction with cytoskeletal cytokeratins (19). To examine this possible interaction, microscopy and subcellular fractionation of a cytokeratin-negative cell line A2780 was compared to cytokeratin positive MCF-7 cells. Microscopy of A2780 (Figure 4.4A) reveals the same pattern of cytoplasm-to-punctate pattern seen in other cell lines transfected with EGFP-ERLBD in the presence of fulvestrant. Subcellular fractionation of untransfected (naked DNA only) versus transfected was performed to rule-out possible upregulation of cytokeratins by Lipofectamine reagent alone (Figure 4.4B). Lipofectamine transfection did not result in any upregulation of CKs 8/18 in cytoplasmic (CP) or cytoskeletal (CS)

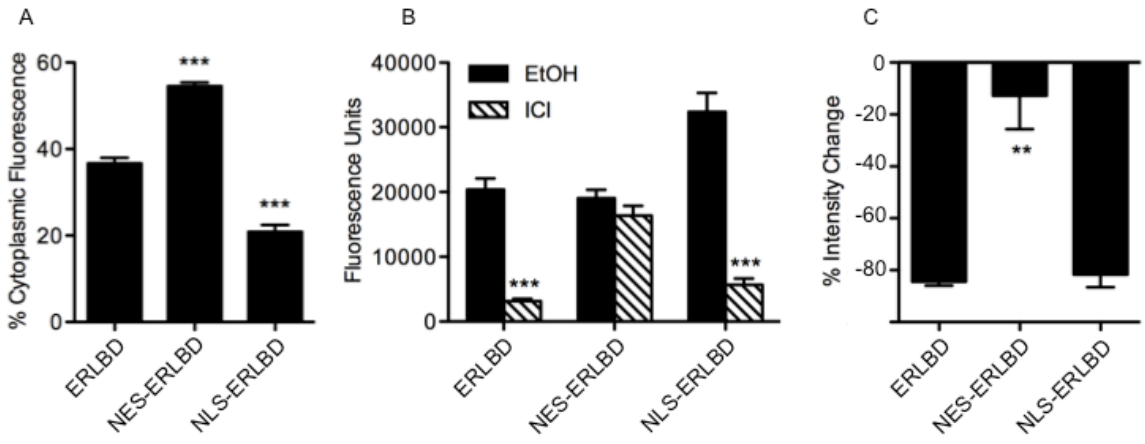

Figure 4.3. Image analysis of fluorescence intensity. Fluorescence intensity analysis in the cytoplasmic and nuclear compartment of 1471.1 cells. A: Fusion of an NES (NES-ERLBD) significantly increased ($p < 0.001$) the fluorescence intensity in the cytoplasm compared to ERLBD. The fusion of an NLS (NLS-ERLBD) significantly decreased the fluorescence intensity in the cytoplasm (meaning an increase in nuclear intensity) compared to ERLBD, $p < 0.001$. B: Quantitation of fluorescence intensity displacement when treated with fulvestrant 100 nM for 1 hour. ERLBD and NLS-ERLBD showed significant displacement when bound to fulvestrant compared to EtOH treatment, $p < 0.001$. NES-ERLBD did not show significant displacement, $p > 0.05$. C: Percent change of fluorescence intensity after fulvestrant addition (negative values represent loss of fluorescence in defined region of interest). The response to fulvestrant was robust in ERLBD and NLS-ERLBD transfected cells, with a high intensity change of greater than 80%. The response by NES-ERLBD to drug was significantly lower compared to ERLBD and NLS-ERLBD, $p < 0.01$.*** = $p < 0.001$, ** = $p < 0.01$.

fractions (Figure 4.4B, Lipo +/-). MCF-7 cells are known to be cytokeratin positive, and these cells did demonstrate cytokeratins in both untransfected and transfected cells. Knowing the cytokeratin status of these cells, the subcellular fraction of EGFP-ERLBD was evaluated 1 hour after ethanol (ICI -) or fulvestrant 100 nM (ICI +) treatment conditions (Figure 4.4C). In both A2780 and MCF-7 cells, EGFP-ERLBD resided in the cytoplasm (Figure 4.4C, CP, -) when induced with vehicle. However, fulvestrant induced a change in subcellular location to the cytoskeleton (Figure 4.4C, CS, +). Hence we conclude that the cytokeratin status does not affect the subcellular location of EGFP-ERLBD bound to fulvestrant.

Cytokeratin 18/ERLBD Colocalization

To further assess the interaction of the ERLBD truncation with cytokeratins, indirect immunofluorescence of CK18 (red channel) on fixed MCF-7 cells transiently transfected with ERLBD (green channel) was performed. A single plane of each cell was captured in both red and green channels (Figure 4.5). The distribution of ERLBD was similar to that seen in Figure 4.1, with whole-cell distribution after ethanol treatment, and punctate distribution after fulvestrant treatment Figure 4.5A, top and bottom panels). CK18 as visualized by indirect immunofluorescence revealed a typical cytoskeletal interconnected filament pattern (Figure 4.5B, top and bottom panels). The images were pseudocolored with red or green, and merged to visualize colocalization. The merged images revealed little yellow color—which denotes overlapping fluorescence in the red and green channels, so a Colocalization Colormap plugin was utilized in ImageJ to better visualize truly overlapping pixels, and JACoP plugin was used to quantify the colocalization by the Costes' coefficient, which quantifies the colocalization of two fluorophores by statistically correlating

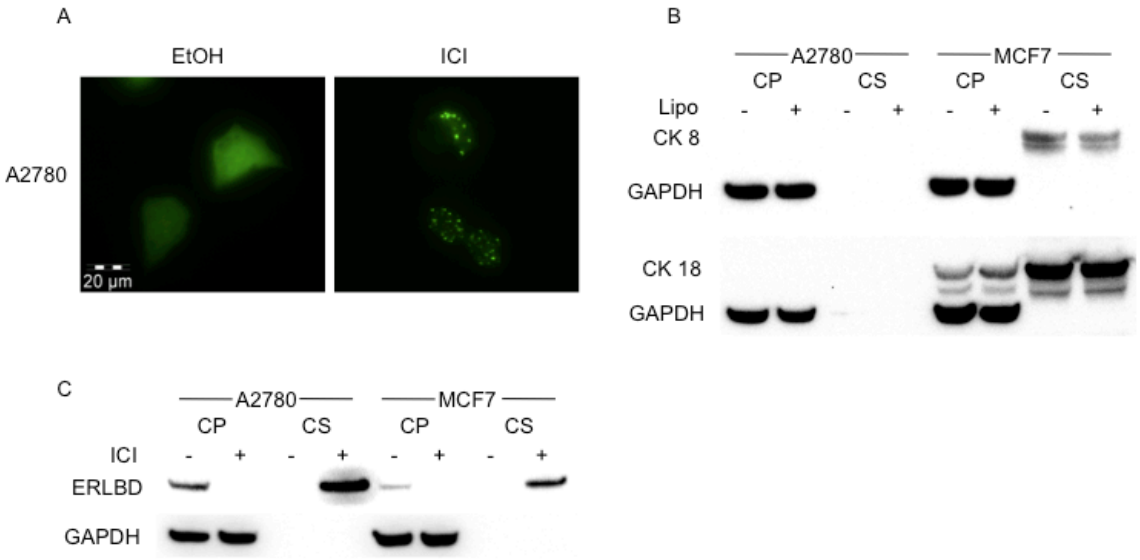

Figure 4.4. Comparison of cytokeratin positive and negative cells. A: Fluorescence microscopy images of A2780 cells transfected with EGFP-ERLBD, and treated with EtOH or fulvestrant 100 nM for 1 hour. B: Western blot after subcellular fractionation of A2780 and MCF-7 cells, with (+) or without (-) transfection by lipofection. Cytokeratins (CK) 8 and 18 were only detected in MCF-7 cells, regardless of transfection status, and appeared only in the cytoskeletal fraction (CS), not the cytoplasmic fraction (CP) while no CK8 or CK18 were detected in A2780. GAPDH was used as a marker of cytoplasmic proteins. C: ERLBD was detected in the cytoplasmic fraction (CP) when treated with ethanol (-), but was seen exclusively in the cytoskeletal fraction (CS) after fulvestrant treatment (+). This response was visible in both A2780 and MCF-7 cells.

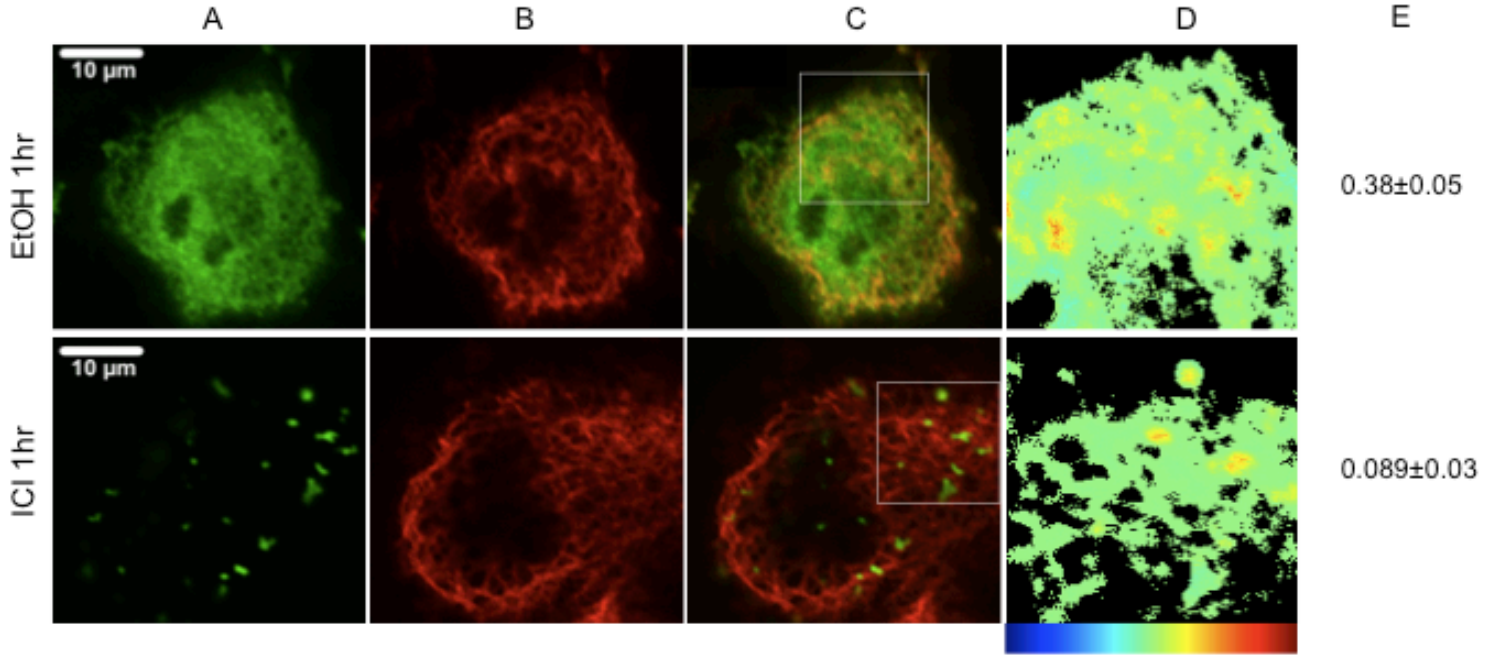

Figure 4.5. Confocal images of indirect immunofluorescence. Indirect immunofluorescence confocal microscopy images of MCF-7 cells transfected with EGFP-ERLBD, stained with anti-cytokeratin 18. A: Location of EGFP-ERLBD with ethanol or fulvestrant treatment. B: Cytokeratin 18 in both treatment conditions. C: Merged image of both green and red channels. Overlapping red and green pixels appear as yellow. D: Expanded region from C, depicted as a colocalization heatmap, with high degrees of pixel color and intensity overlap as dark red, low degrees as blue. E: Pearson's corrected Costes' Coefficient, a quantitation of protein colocalization by JACoP plugin (ImageJ). Significantly colocalized proteins have coefficients greater than 0.5. Significant colocalization was not seen with ethanol or fulvestrant treatment.

the overlap and pixel intensity of each color (35). The Costes' coefficient predicts colocalized fluorophores for values over 0.5. High degrees of pixel intensity overlap are depicted in the colormap by red and brown colors, where low degrees of overlap are depicted as blue and green (Figure 4.5D, top and bottom panels). ERLBD treated with ethanol had a Costes' coefficient of 0.38 ± 0.05; whereas with fulvestrant treatment yielded a coefficient of 0.089±0.03. Neither treatment condition suggests a significant colocalization of the ERLBD with cytokeratin 18.

Induced Sequestration of ERLBD-bearing Protein

To test the ability of ERLBD to control a protein's localization, and therefore function, we subcloned a mutated coiled-coil (CCmut3) motif from Bcr-Abl previously developed in our lab (8). When introduced into Bcr-Abl positive CML cells, this coiled-coil disrupts signaling by Bcr-Abl and induces apoptosis. We hypothesized that ERLBD-CCmut3, when bound to fulvestrant, would cause sequestration of CCmut3 thereby preventing the disruption of Bcr-Abl signaling and nullifying its apoptotic response. Microscopy studies reveal that fulvestrant does indeed sequester ERLBD-CCmut3 similarly to ERLBD alone in K562 cells, whereas ER agonist estradiol and partial agonist tamoxifen did not induce protein translocation (Figure 4.6A). To test the decrease in apoptotic potential of ERLBD-CCmut3 sequestered by fulvestrant, flow cytometry with 7AAD staining was performed. Bcr-Abl positive K562 CML cells were transfected with EGFP, EGFP-ERLBD, or EGFP-ERLBD-CCmut3. These cells were treated with ethanol or fulvestrant at escalating doses for 48 and 72 hours; untransfected cells were treated with Gleevec® as a positive control (Figure 4.6B). EGFP with or without fulvestrant demonstrated low 7AAD staining, with 2.2% ± 0.4 and 1.8% ± 0.4 respectively. EGFP-ERLBD trended toward

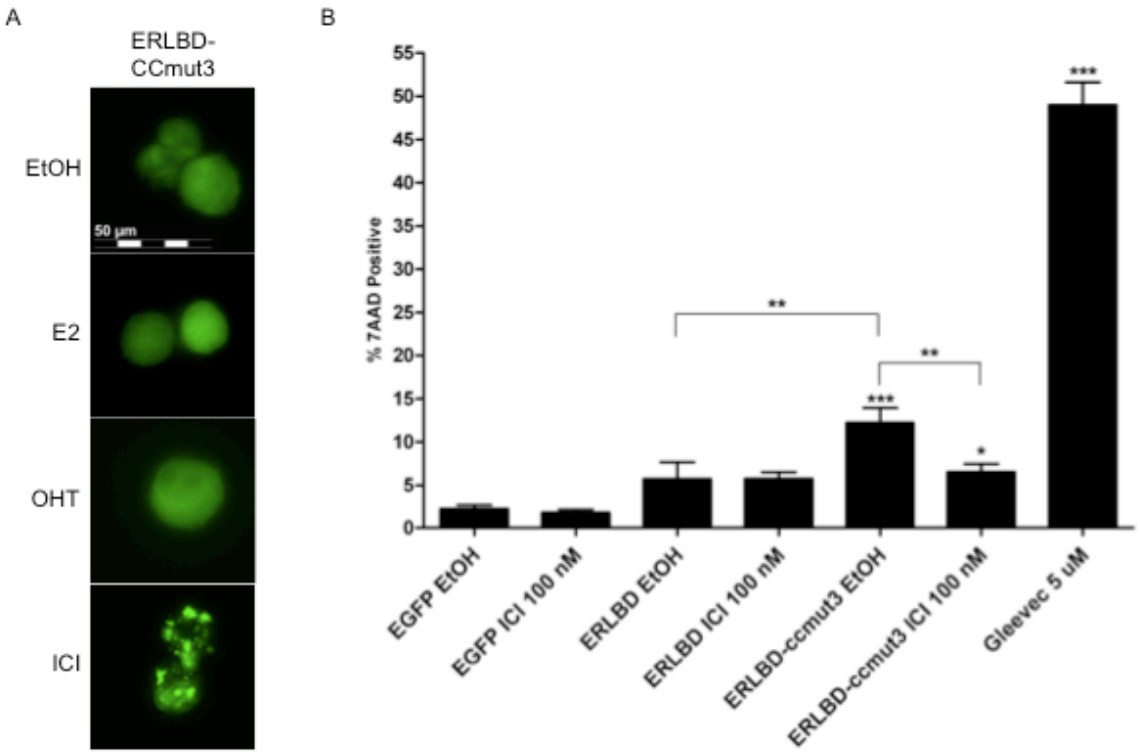

Figure 4.6. ERLBD-CCmut3 in K562 cells. A: Fluorescence microscopy of K562 cells transfected with EGFP-ERLBD-CCmut3 and treated with either ethanol (EtOH), estradiol (E2) 100 nM, tamoxifen (OHT) 100nM, or fulvestrant (ICI) 100nM for 1 hour. Fulvestrant was the only ligand causing appreciable protein translocation. The same subcellular distribution pattern was demonstrated when treated with fulvestrant, despite the inclusion of a functional peptide capable of binding an endogenous protein. B: 7AAD staining as detected by flow cytometry, as a percent of cell population. Only cells transfected with ERLBD-CCmut3 showed significantly higher 7AAD staining than the negative control (EGFP). ERLBD-CCmut3 treated with ethanol demonstrated the highest staining of transfected cells, significantly higher than EGFP, $p < 0.001$. When bound to fulvestrant, however, 7AAD staining significantly decreased, $p < 0.01$. Untransfected cells were treated with Gleevec® as a positive control for 7AAD staining. *** = $p < 0.001$, ** = $p < 0.01$, * $p < 0.05$.

higher 7AAD staining, though not statistically significant with either ethanol or fulvestrant treatment: 5.7% ± 1.9, and 5.7% ± 0.8, respectively. The construct with CCmut3 significantly increased 7AAD staining compared to EGFP as expected due to disruption of Bcr-Abl signaling, but only when treated with ethanol (vehicle), 12.2% ± 1.7, p <0 .001. When treated with fulvestrant, the sequestration of CCmut3 allowed Bcr-Abl to continue its proliferative effects, with decreased apoptosis as measured by 7AAD to 5.8% ± 0.8 (significantly different from ethanol treatment, p < 0.01). The results were consistent at 48 (data not shown) and 72 hours of treatment. Fulvestrant was also tested at higher doses (400 nM and 800 nM), though these were not significantly different from 100 nM (data not shown).

Discussion

Our lab has previously had success in creating novel "protein switches" capable of ligand-dependent subcellular translocation of exogenous proteins. To our knowledge, this is the first paper to describe harnessing the ligand-inducible translocation of the estrogen receptor ligand-binding domain (ERLBD) to generate a protein switch capable of controlled movement from the cytoplasm, and/or nucleus to the insoluble cytoskeletal fraction. This controlled translocation is accomplished when the LBD binds the synthetic antagonist fulvestrant. The estrogen receptor (ER) is a protein that typically resides in the nucleus, but when bound to the SERD fulvestrant, is found in insoluble clusters in both the nucleus and cytoplasm (14, 15). Here, we showed that removing the ligand-binding domain (LBD) from the full-length receptor still responds to fulvestrant, but that its localization can be controlled by the lack-of or inclusion-of signal sequences. Microscopy studies revealed that the inclusion of a nuclear localization signal (NLS) directed the protein

to the nucleus before ligand, and maintained the nuclear location while forming insoluble aggregates upon ligand addition. Similarly, fusing a nuclear export signal (NES) to the LBD directed the protein to the cytoplasm. This control of protein localization was demonstrated in a wide array of cell types, including those with or without cytokeratins 8 and 18, and cancer or non-cancer types. Previously, the response of the ER to fulvestrant was attributed to its interaction with cytokeratins 8 and 18 in both the cytoplasm and the nucleus (19), and this was also claimed to be an intermediate step toward proteasomal degradation when bound to ligand (13, 19). Our subcellular fractionation in MCF-7 cells (cytokeratin 8 and 18 positive) *and* A2780 (cytokeratin 8 and 18 negative) showed that the ERLBD fusion protein is capable of translocation from the cytoplasm to the insoluble cytoskeletal fraction regardless of cytokeratin status. Confocal microscopy confirmed that colocalization of ERLBD with cytokeratins was minimal, and played no role. Perhaps in other studies, localization to CK 8 and 18 may be mediated by other domains of the ER not present in our ERLBD constructs.

The utility of this controlled localization was demonstrated by fusing the ERLBD protein to a functional peptide (CCmut3) that had previously been shown to bind to and disrupt Bcr-Abl signaling in K562 cells and induce apoptosis (8). We hypothesized that after fulvestrant addition, ERLBD-CCmut3 protein would translocate away from Bcr-Abl to insoluble clusters, and would relieve the Bcr-Abl signaling inhibition. Indeed, despite the fact that CCmut3 interacts with Bcr-Abl, microscopy studies corroborated that ERLBD-CCmut3 formed insoluble clusters with the addition of fulvestrant, indicating ligand-inducible controlled localization of CCmut3. Lastly, ERLBD-CCmut3 showed apoptosis without fulvestrant, but a significant drop in apoptosis when bound to drug, confirming our hypothesis of

location altering function. This technology has the potential to be used as a controlled tool to test and verify synthetic exogenous protein or peptide function, as demonstrated here, but may also be useful to induce an *in trans* sequestration of an endogenous protein target. Any protein binding domain can easily be cloned into an ERLBD vector, thus allowing a large variety of potential cellular targets, in multiple cell types.

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CHAPTER 5

CONTROLLED ACCESS OF P53 TO THE NUCLEUS REGULATES ITS PROTEASOMAL DEGRADATION BY MDM2

Abstract

The tumor suppressor p53 can be sent to the proteasome for degradation by placing its nucleo-cytoplasmic shuttling under ligand control. Endogenous p53 is ubiquitinated by MDM2 in the nucleus, and controlling the access of p53 to the nuclear compartment regulates its ubiquitination and proteasomal degradation. This was accomplished by the use of a "protein switch" that places nuclear translocation under the control of externally applied dexamethasone. Fluorescence microscopy revealed that sending protein switch p53 (PS-p53) to the nucleus produces a distinct punctate distribution in both the cytoplasm and nucleus. The role nuclear access was investigated by inhibiting classical nuclear export with leptomycin B. Trapping PS-p53 in the nucleus only allows this punctate staining in that compartment, suggesting that PS-p53 must translocate first to the nuclear

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[$]Both authors contributed equally to this work, and are considered co-first authors. JRD and MM were both involved in the conceptualization and execution of this work. MM created and tested full-length p53, JRD tested full-length and truncated versions of p53. CSL provided intellectual contribution.

compartment for cytoplasmic punctate staining to occur. The role of MDM2 binding was explored by inhibiting MDM2/p53 binding with nutlin-3. Inhibition of this interaction blocked both nuclear export and cytoplasmic and nuclear punctate staining, providing evidence that any change in localization after nuclear translocation is due to MDM2 binding. Further, blocking the proteolytic activity of the proteasome maintained the nuclear localization of the construct. Truncations of p53 were made to determine smaller constructs still capable of interacting with MDM2, and their subcellular localization and degradation potential was quantified. PS-p53 and a smaller construct, construct containing the two MDM2 binding regions, Box I+V, were indeed degraded by the proteasome as measured by loss of enhanced green fluorescent protein that was also fused to the construct. The influence of these constructs on p53 gene transactivation function was assessed, and revealed that PS-p53 decreased gene transactivation, while PS-p53(BoxI+Vmut) did not significantly change baseline gene transactivation.

Introduction

Protein subcellular localization is tightly regulated in order for cells to control their function. Protein mislocalization has been implicated in a number of diseases, (1) and it has been proposed that correcting this mislocalization may be a potential treatment option (1, 2). It has also been found that inducing a change in a protein's localization can be used to prevent that protein from functioning (3), or can exploit that protein's activity in a different compartment (4). In an effort to control protein localization, our lab has developed technologies aimed at altering location and function (3-8). Recently, we have shown that a protein's function can be altered if it is sequestered in cytoskeletal aggregates (3), or that apoptosis can be induced by

sending p53 to the mitochondria (9). One of the technologies we have developed is the cytoplasm-to-nucleus protein switch (PS). This PS contains a nuclear export signal, a nuclear localization signal, and a ligand-inducible domain from the glucocorticoid receptor (GR). Ligand binding controls localization by a dexamethasone-specific ligand-binding domain (LBD) from GR, such that when expressed the protein is localized to the cytoplasm but is targeted to the nucleus upon induction by the ligand dexamethasone (dex) (7, 8, 10).

p53 is a tumor suppressor that is maintained at low levels in normal cells, but rapidly accumulates in the nucleus in response to stress, such as DNA damage, hyperproliferation, chemotherapeutic agents, ultraviolet light, and hypoxia (11-15). The half-life of p53 is 6-20 minutes in healthy cells. However the concentration of p53 is increased 3- to 10-fold and the half-life is improved to hours in response to stress (11, 12). The accumulation is due to two factors: induced transcription of the *TP53* gene and reduced ubiquitination and proteasomal degradation (16). The accumulated p53 proteins are able to activate genes that promote growth arrest, apoptosis, and DNA repair through its transactivation function (17), and by inhibiting p53's degradation (18). MDM2 (HDM2 in humans) is the main protein responsible for the maintenance of p53, and does so with its RING finger domain E3 ligase activity that coordinates the transfer of ubiquitin from an E2 enzyme to lysine residues within p53 (16, 19-24). Proteasomes recognize ubiquitinated proteins and degrade them (25, 26). However, overexpression of MDM2 results in downregulation of p53 and may lead to cell hyperproliferation, and this has been implicated in a variety of human cancers (27, 28). Small molecule inhibitors of the MDM2-p53 interaction have been developed to restore p53 activity, and may provide significant clinical benefit (29-36).

Though the field of study of MDM2 and p53 is immense, the exact process of ubiquitination and subsequent degradation has not been fully elucidated. It is clear that conformation of p53, and the relative concentration of MDM2 to p53 greatly influence the ubiquitination fate of p53 (21, 37). It is also known that MDM2 and p53 shuttle back and forth between the cytoplasm and nucleus via their respective NES and NLS, but that ubiquitination of p53 by MDM2 is mostly localized within the nucleus, and that access to both compartments is necessary for degradation (38). MDM2 can monoubiquitinate or polyubiquitinate p53; monoubiquitination exposes an NES in the C-terminal region of p53 and results in nuclear export, possibly with MDM2 in tow (37, 39). In the cytoplasm, MDM2 can efficiently polyubiquitinate p53, but it requires p53 to be in tetrameric form. Polyubiquitination can occur in the nucleus, and may favor proteasomal degradation within the nucleus (37).

In this work, we hypothesized that p53 fused to our cytoplasm-to-nucleus protein switch could provide for a controlled interaction of p53 with MDM2, and thereby control the proteasomal degradation by ligand induction. We found that indeed controlling the access of p53 to the nucleus limited the apparent interaction of p53 to MDM2 to ligand induction, and we found this to be dependent upon nuclear translocation, nuclear export, and a functioning proteasome, with the ultimate outcome of proteasomal degradation of our construct. We also found that smaller domains of p53 could be fused to the protein switch with similar outcomes. To our knowledge, this is the first time that the interaction of p53 with MDM2 has been controlled by ligand induction, and represents an interesting method of targeting a protein to the ubiquitin-proteasome pathway.

Materials and Methods

Construction of EGFP-PS-p53

The DNA encoding p53 was amplified through PCR from pCMV-p53 wt (a generous gift from Dr. S. J. Baker, Addgene, Cambridge, MA) using the primers 5′-GCGCGCGGATCCGCCATGGAGGAGCCGCAGT-3′ and 5′-GCGCGCGGATCCT-CAGTCTGAGTCAGGCCCTTCTGTC-3′. This was subcloned into the BamHI restriction enzyme site in the EGFP-HIV-MycA8-GRLBD (EGFP-PS) plasmid constructed previously (7). The EGFP-PS contains a nuclear export signal (NES) from HIV-rev protein (8), a nuclear localization signal (NLS) from MycA8 protein (7) and a ligand binding domain from glucocorticoid receptor (GRLBD) with a point mutation (C656G) that makes it 10 times more sensitive to the agonist dexamethasone (dex) (40).

Construction of Modified EGFP-PS-p53 Plasmids

EGFP-PS-p53ΔMBD: p53 was amplified without its MDM2 binding domain (MBD) through PCR using 5′-GCGCGCGCGCGGTACCGCTCCCAGAATGCCAGA-GGC-′3 and 5′-GCGCGCG-GATCCTCAGTCTGAGTCAGGCCCTTCTGTC-3′ primers. This was subcloned into the KpnI and BamHI restriction enzyme sites in EGFP-PS (40).

EGFP-PS-p53ΔC: p53 was amplified without its C-terminal region by PCR using 5’- AATAATCTCGAGTTATGGAGGAGCCGCAGTCAG-3’ and 5’- TAATAAC-TGCAGCCCAGCCTGGGCATCCTTG-3’ primers, which introduce XhoI and PstI restriction enzyme sites. After PCR, the fragment and EGFP-PS plasmid were digested with these enzymes, and ligated together.

EGFP-PS-p53ΔTD: To remove the tetramerization domain (TD) the pre- and post- TD fragments were spliced together. The pre-TD fragment of p53 was amplified by PCR using '5- AATAATCTCGAGTTATGGAGGAGCCGCAGTCAG-3' and 5'- TACGAACTGCAGTGGTTTCTTCTTTGGCTGG-3' primers, which introduce XhoI and PstI restriction sites. The post-TD domain was amplified with 5'-AATAATGGTACCTAAGGAGCCAGGGGGGAGCAG-3' and 5'- TAATAAGGGCC-CGTCTGAGTCAGGCCCTTCTG-3' primers, which introduce KpnI and ApaI restrictions sites. The fragments and plasmids were digested and ligated with their respective enzymes, and ligated together in a two-step process.

EGFP-PS-p53(Box I) and (Box I + V): DNA oligonucleotides were synthesized by the DNA/Peptide Core Facility at the University of Utah. For Box I the sense and antisense fragments were 5'-TCGAGTTGAGCCCCCTCTGAGTCAGGAAACA-TTTTCAGACCTATGGAAACTACTTCCTGAAAACAACCTGCA-3' and 5'-GGTTG-TTTTCAGGAAGTAGTTTCCATAGGTCTGAAAATGTTTCCTGACTCAGAGGGGGC TCAAC-3'. These oligos were annealed, and ligated between XhoI and PstI in the parent plasmid. For Box V, 5'-CTCGGAACAGCTTTGAGGTGCGTGTTTGTGCC-TGTCCTGGGAGAGACGGGCC-3' and 5'- CGTCTCTCCCAGGACAGGCACAAA-CACGCACCTCAAAGCTGTTCCGAGGTAC-3' were annealed, and ligated between KpnI and ApaI restriction sites in the parent plasmid.

F270A mutations: All template plasmids had the F270A mutation introduced using the QuickChange II Site Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) with forward primer 5'- CTACTGGGACGGAACAGCGCT-GAGGTGCGTGTTTGTG-3' and reverse primer 5'-CACAAACACGCACCTCAG-CGCTGTTCCGTCCCAGTAG-3'.

The University of Utah Core DNA Sequencing Facility verified the sequences of all subcloned constructs.

Cell Lines and Transient Transfections

C127-derived murine mammary carcinoma cells (1471.1 cells)(41) (gift of G. Hager, NCI, NIH) were grown as monolayers in DMEM (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (Invitrogen), 1% penicillin-streptomycin-glutamine (Invitrogen), and 0.1% gentamicin (Invitrogen). The cells were maintained in a 5% CO_2 incubator at 37ºC. 7.5×10^4 cells were seeded in 2-well live cell chambers (Nalgene Nunc, Rochester, NY). Transfections were carried out 24 hours after seeding using 1 pmol DNA and Lipofectamine 2000 (Invitrogen) following the manufacturer's recommendations. Constructs were induced with 100 nM of the dex ligand, 24 hours after transfection for 1 hour, or as otherwise noted (7).

Fluorescence Microscopy

Approximately 24 hours after transfection, protein localization was viewed by fluorescence microscopy. For microscopy studies, cells were plated on a clear cover glass in six well plates (Greiner Bio-One Cellstar, Monroe, NC) or live cell chambers (Nalge Nunc, Rochester, NY). Prior to cell imaging, media was replaced with phenol red-free DMEM (Invitrogen) containing 10% charcoal-stripped fetal bovine serum (CS-FBS, Invitrogen). Images were acquired as previously,(4) using an Olympus IX71F fluorescence microscope (Scientific Instrument Company, Aurora, CO) with high-quality narrow band GFP filter (ex: HQ480/20 nm, em: HQ510/20 nm) from Chroma Technology (Brattleboro, VT) with a 40X or 60X PlanApo oil immersion objectives (NA 1.00) on an F-View Monochrome CCD camera. For live-cell imaging,

the microscope stage was maintained at 37°C with an air stream incubator (Nevtek ASI 400, Burnsville, VA). All experiments were repeated in triplicates (n=3) and 10 cells were analyzed for each time-point in each experiment. Green fluorescent images in this publication have been pseudocolored with ImageJ software, and brightness and contrast have been adjusted for visibility.

Treatment with LMB, Nutlin-3, and MG132

Cells were incubated with 10 nM leptomycin B (LMB, an inhibitor of nuclear export) 10 hours after transfection and 14 hours before ligand induction with dex followed by microscopy. Another population of transfected cells was incubated with 10 μM nutlin-3 1 hour before ligand induction (Sigma, St. Louis, MO). MG132 (Sigma) was used at a concentration of 50 μM.

Flow Cytometry for GFP Intensity

MCF-7 cells were treated with 10 mg/mL cycloheximide 24 h after transfection to inhibit protein synthesis (42). Cells were then treated with 100nM dex for 0, 2, 4, and 6 h. After treatment, cells were suspended in PBS and analyzed using the FACSCanto-II (BD-BioSciences, University of Utah Core Facility) and FACSDiva software (43). Excitation was set at 488 nm and detected at 507 for EGFP intensity. The means from three separate experiments (n=3) were analyzed using two-way ANOVA with Sidak's multiple comparisons post-hoc test.

Reporter Gene Assay

As previously (9), 3.5 μg of each construct was co-transfected with 0.35 μg of pRL-SV40 plasmid encoding for *Renilla* luciferase (Promega, Madison, WI) as well

as 3.5 µg of p53-Luc Cis-Reporter (Agilent Technologies, Santa Clara, CA) in MCF-7 cells. Cells were then treated with 100nM dex to be compared to no dex treated cells. *Firefly* and *Renilla* luminescence were detected via the Dual-Glo Luciferase Assay System (Promega) per manufacturer's instructions using the PlateLumino Luminometer (Stratec Biomedical Systems, Birkenfeld, Germany). The *Firefly* luciferase activity was normalized with *Renilla* luciferase values. The positive control (E-p53) was set at 100% and untransfected cells were set at 0%. The experiment was performed with an n=3 and analyzed using two-way ANOVA with Bonferroni's post-hoc test.

Results

p53 Fused to the Proteasome Switch Localizes to the Cytoplasm after Nuclear Import and Export

Our previous studies have shown that the localization of exogenous proteins can be controlled by fusing a protein switch (PS) containing an NES, NLS, and ligand-binding domain (LBD) (2, 4, 7). In this study, we aimed to rationally design a protein switch that would target the proteasome; in the absence of ligand, it would localize in the cytoplasm, but after ligand addition (dexamethasone; dex), it would target the proteasome. We hypothesized that a PS-p53 fusion would target the proteasome when p53 was allowed to interact with MDM2, which is mainly localized within the nucleus.

The p53 cDNA was subcloned into the protein switch (PS) to create E-PS-p53. The enhanced green fluorescent protein (EGFP; E) tag facilitates localization tracking with fluorescence microscopy. After transient transfection in 1471.1 mouse mammary adenocarcinoma cells, the "empty" protein switch (E-PS) protein localizes

in the cytoplasm (Figure 5.1 A, top left), but translocates into the nucleus after dex addition (Figure 5.1 A, top right). Wild-type p53 tagged to EGFP is unaffected by dex treatment, and appears to localize mostly in the nucleus and in cytoplasmic clusters (Figure 5.1 A, middle). The localization of transiently transfected p53 (E-PS-p53) was examined (Figure 5.1 A, bottom panel) before and after dex treatment. Before drug, the protein had a mostly cytoplasmic localization, similar to E-PS. However within an hour of dex treatment, the protein took on a distinct punctate distribution in both the cytoplasm and nucleus.

E-PS-p53 was transiently transfected in human breast cancer cell lines MCF-7, T-47D, and MDA-MB-231 (Figure 5.1 B). E-PS-p53 appeared to follow the same response pattern to dex in the human breast cancer cells. However, ligand responsiveness was not observed in HeLa cells; the localization did not change with dexamethasone addition (data not shown).

PS-p53 Interacts with MDM2 in the Nucleus Before Export

To investigate the mechanism behind the cytoplasmic and nuclear punctate localization of E-PS-p53, the effect of drug inhibitors of nuclear export (leptomycin B; LMB) and MDM2 interaction (nutlin-3) were examined. LMB is an inhibitor of CRM1 (chromosome region maintenance/exportin 1), which mediates classic nuclear export via nuclear export signals, including the nuclear export of wild-type p53 (1, 44, 45). By inhibiting export, proteins localized within the nucleus are trapped there, but proteins not located—or shuttling to—the nucleus will be unaffected. The

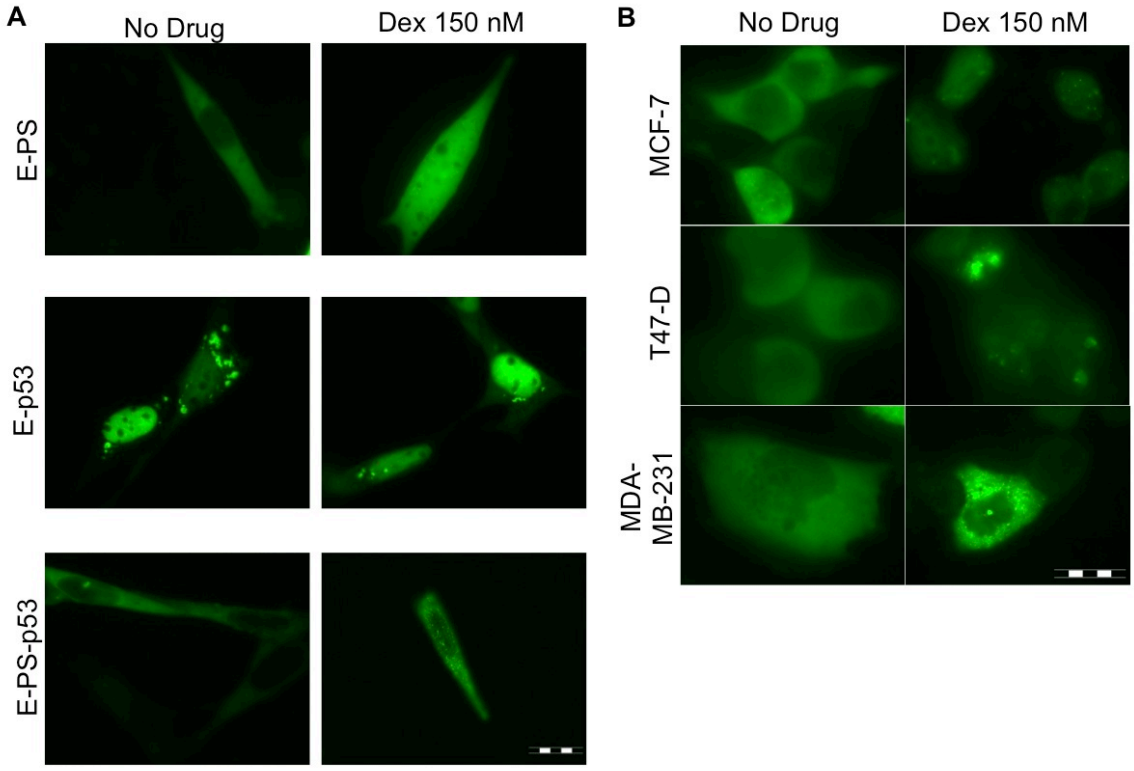


Figure 5.1. Localization of PS-p53 in breast cancer cells. A: Fluorescence microscopy of transiently transfected 1471.1 cells, with no drug treatment (left) and 150 nM dexamethasone (dex) at 1 hour (right). The protein switch, E-PS, localized in the cytoplasm without drug, but translocated to the nucleus after dex addition. Wild-type p53 tagged to EGFP (E-p53) did not respond to dex treatment, and had similar localization regardless of drug treatment. The protein switch fused to p53 (E-PS-p53) localized in the cytoplasm without dex, but demonstrated a distinct punctate pattern in the cytoplasm and nucleus. B: E-PS-p53 transiently transfected in various cancer cell lines (MCF-7, T47D, MDA-MB-231), with and without 1 hour of 150 nM dex, showing similar results to 1471.1 cells. Scale bars in A and B represent 20 μm.

results of a 14 hour preincubation with 10 nM LMB, followed by 1 hour incubation with 150 nM dex, demonstrate that E-PS-p53 accumulates within the nucleus, showing the same punctate distribution only in the nucleus (Figure 4.2 A). The accumulation is due to the unavailability of the nuclear export machinery to shuttle the construct back to the cytoplasm. This confirms that the protein translocates to the nucleus upon dex treatment, before shuttling back to cytoplasm.

MDM2 is the main E3 ubiquitin ligase for p53, and it resides mainly in the nucleus. We already determined that E-PS-p53 first travels to the nucleus after dex addition (Figure 5.1), so we speculated that the subsequent nuclear export and punctate distribution was due to MDM2 by facilitating ubiquitin conjugation to the p53 domain of our construct. Adding nutlin-3, a competitive inhibitor of the interaction of p53 with MDM2, elucidated the role of MDM2 in our system. Figure 4.2 B shows the results of nutlin-3 incubation in 1471.1 cells transfected with E-PS-p53, with or without dex addition. Without dex (left image), but with nutlin-3, the localization is no different from that seen without nutlin-3 incubation; the protein is localized mainly in the cytoplasm. However, dex induction in the presence of nutlin-3 (right image) demonstrates nuclear translocation but lacks any evidence of nuclear export, and the protein does not show punctate staining. The localization with nutlin-3 appears similar to E-PS with dex alone—the p53 domain does not appear to have an impact on localization. Taken together, these results suggest that E-PS-p53 responds to dex treatment by translocating to the nucleus where it interacts with MDM2, and subsequently shuttles back to the cytoplasm and takes on punctate distribution.

Inhibiting the Proteasome Alters PS-p53 Localization

Wild-type p53 is sent to the ubiquitin-proteasome pathway by MDM2. To investigate whether the cytoplasmic and nuclear clusters were indeed constructs destined for the proteasome, we tested the effect of proteasomal inhibition on EGFP-PS-p53 localization. 1471.1 cells were again transiently transfected with EGFP-PS-p53 and incubated with the proteasomal inhibitor MG132 ± dex. MG132 did not appear to have an effect on the localization of the construct in the absence of dex (Figure 5.2 C, left image), but in the presence of dex, the construct demonstrated nuclear accumulation without visible clusters in the nucleus or cytoplasm (Figure 5.2 C, right image). Thus, a functioning proteasome was necessary for the nuclear export and punctate distribution of E-PS-p53 after dex treatment.

p53 Truncations Also Interact with MDM2 in the Nucleus

We sought to determine if smaller constructs comprised of p53 domains could produce the same localization change in response to ligand when fused to the protein switch. Figure 5.3 A shows representative images of select p53 truncations. A table of all tested constructs is shown in Figure 5.3 B. All constructs had varying degrees of cytoplasmic and nuclear localization upon dex addition, and some constructs did not result in proteasome pathway targeting. The constructs that did yield localization control similar to E-PS-p53 were E-PS-p53ΔC, E-PS-p53ΔMBD, and E-

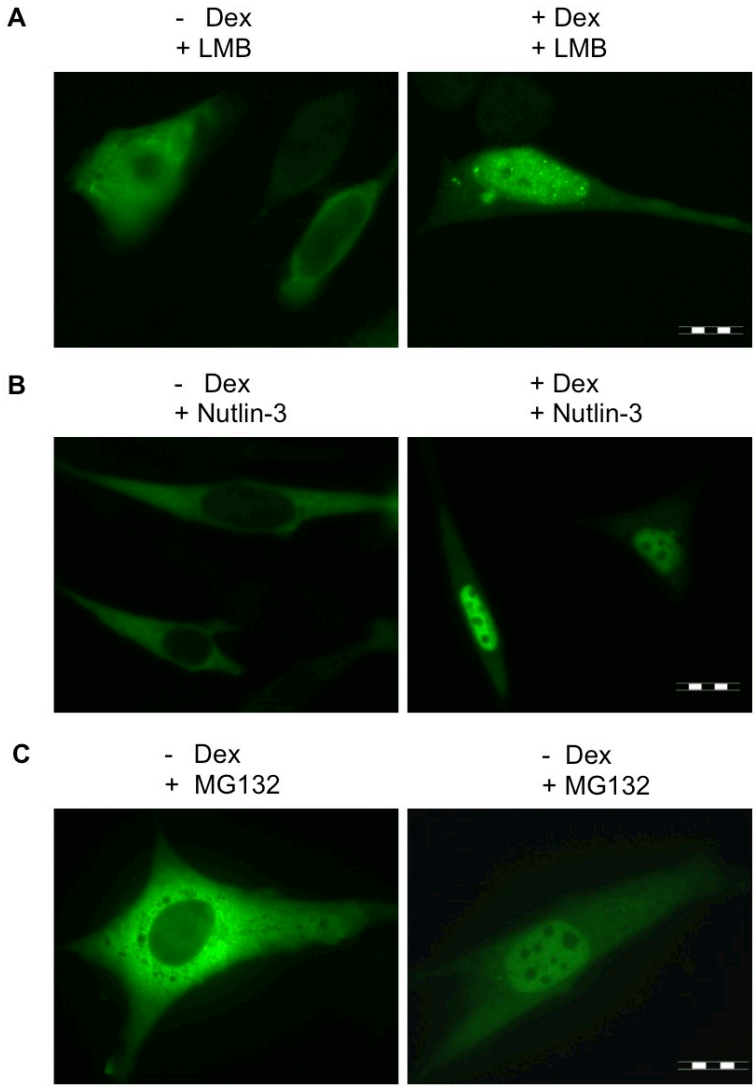
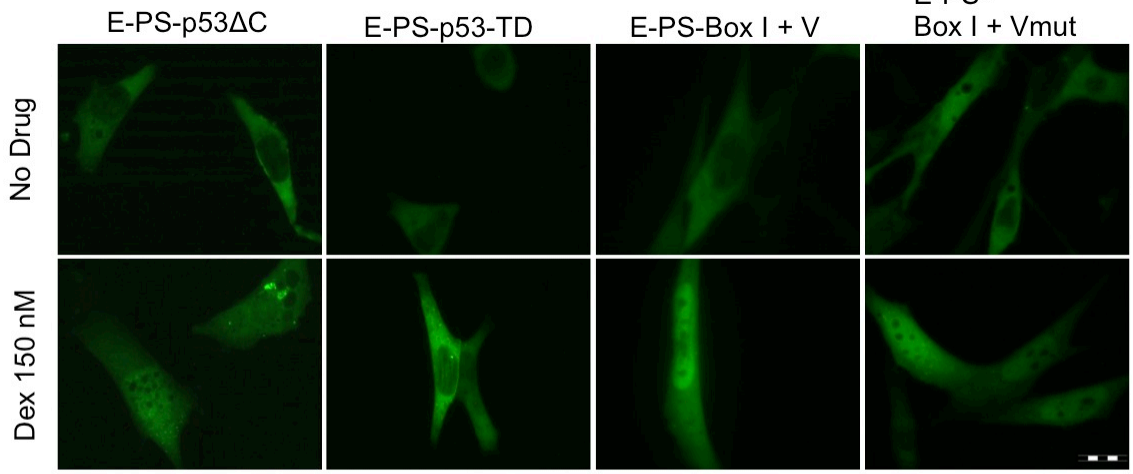


Figure 5.2. Mechanism of PS-p53 localization. The effect of inhibition of nuclear export or MDM2 was examined in 1471.1 cells transiently transfected with E-PS-p53. A: Cells incubated with LMB, an inhibitor of nuclear export, showed a cytoplasmic localization before dexamethasone treatment (left), but constructs demonstrated nuclear accumulation and punctate staining almost exclusively in the nucleus after 1 hour of 150 nM dex (right). B: Cells were incubated with an inhibitor of the interaction between MDM2 and p53. Without dex, nutlin-3 did not significantly change the baseline localization of E-PS-p53, but after 1 hour of 150 nM dex, the construct accumulated in the nucleus and did not show any punctate distribution. C: The role of the proteasome was evaluated by incubating cells with an inhibitor of the proteasome, MG132. The inhibitor had no effect on the baseline localization of the construct, but after 1 hour of incubation with 150 nM dex, the construct took on a mostly nuclear localization, and showed a lack of cytoplasmic or nuclear punctate staining. Scale bars represent 20 μm.

A



B

Construct	Localization	Targeting
E-PS-p53	C + N	Y
E-PS-p53mut*	C >> N	N
E-PS-p53ΔC	C + N	Y
E-PS-p53ΔTD*	N > C	N
E-PS-p53ΔTDmut*	N >> C	N
E-PS-TD	C > N	~
E-PS-p53ΔMBD*	N >> C	Y
E-PS-Box I*	N > C	N
E-PS-Box I + V	N > C	N
E-PS-Box I + Vmut	C + N	Y

* Microscopy not shown

Figure 5.3. Localization of p53 truncations A: Representative fluorescence microscopy images of 1471.1 cells transiently transfected with plasmids encoding truncated p53 constructs fused to the protein switch. The top row is no drug, whereas the bottom row is 1 hour of 150 nM dexamethasone. ΔC, ΔMBD, and Box I+Vmut constructs demonstrated proteasome pathway targeting; the TD construct was equivocal in regard to proteasome pathway targeting. B: Table of all created constructs, their localization after dexamethasone addition, and whether they appeared to target the proteasome pathway. Scale bar represents 20 μm.

PS-Box I+Vmut. E-PS-TD only included the tetramerization domain of p53, and showed equivocal targeting in microscopy studies. Interestingly, removal of the N-terminal MBD domain did not abrogate targeting. The F270A mutation (mut) has been previously described as leading to destabilization and hyperubiquitination and increased proteasomal degradation of p53 beyond the C-terminal region typically associated with polyubiquitination (46). We included this mutation in several constructs to see if it imparted increased targeting to the proteasome pathway. The mutation did not universally increase proteasome pathway targeting, but it did for the Box I+V construct.

The PS-p53 Proteasomal Degradation is Under Ligand Control

To further examine the degradation of the PS-p53 upon ligand addition, MCF-7 cells were transfected with selected constructs followed by dex and cycloheximide treatments. Cycloheximide was used to ensure no additional protein synthesis (42) after the addition of dex to be able to quantify only the loss of GFP intensity (43) before and after dex treatment. Expectedly, the negative controls (EGFP, E-p53, and E-PS-TD) did not show any change in GFP intensity over the 6 hour dex treatment (Figure 5.4). On the other hand, E-PS-p53 and E-PS-Box I+Vmut displayed decreased GFP intensity ($p<0.05$). Similar to the negative controls, the E-PS-ΔC with dex treatment was not degraded despite its punctate formation. E-p53 demonstrated a significant increase in fluorescence intensity ($p<0.05$).

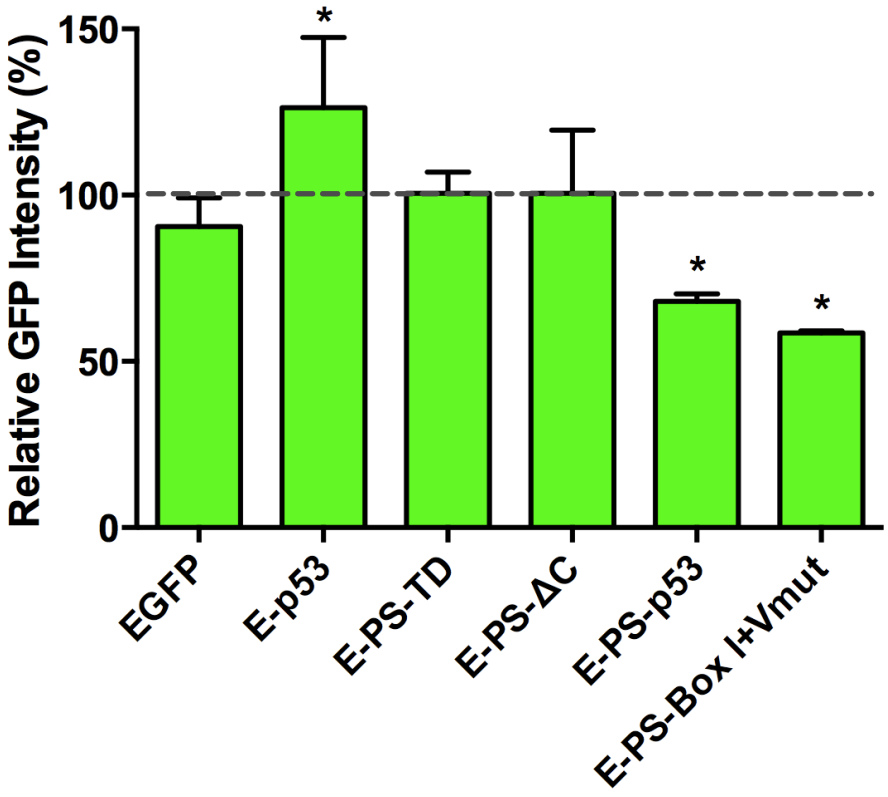


Figure 5.4. Protein degradation by loss of GFP fluorescence intensity. The GFP intensity was measured in transfected MCF-7 cells after 6 hours of dex treatment. The dashed line represents the intensity before drug addition. E-PS-p53 and E-PS-Box I+Vmut were the only constructs showing reduction in relative GFP intensity compared to EGFP control. E-p53 had a significant increase in intensity. Mean values before and after treatment were analyzed using one-way ANOVA with Tukey's multiple comparisons post-hoc test (*p<0.05). Error bars represent standard deviations from three independent experiments (n=3).

PS-p53 Constructs do not Initiate Transactivation Via p53 Promoter

In addition to its proteasomal targeting, we investigated the ability of p53 in the proteasomal switch to act as a transcription factor. Since the p53 cis-reporter system is widely utilized to evaluate the transcriptional activity of p53, MCF-7 cells were used to evaluate the activity of our proteasomal constructs before and after dex (Figure 5.5). The negative controls (EGFP and E-PS) reflected the endogenous transcriptional activity (all cells contain endogenous p53), which is significantly lower than the E-p53 treated cells (positive control). Both E-PS-p53 and E-PS-Box I+Vmut showed no additional activity. Similar to the negative controls, the E-PS-Box I+Vmut did not affect the endogenous transcriptional activity. However, the E-PS-p53, similar to E-PS-TD, showed lower transcriptional activity than the negative control.

Discussion

This work demonstrated that p53 could be fused to a protein switch capable of nucleo-cytoplasmic translocation under the control of external ligand. We took advantage of our nuclear protein switch (PS), which contains a ligand inducible nuclear import (47) and an export signal to control the localization to the nucleus (48). The localization is controlled by a dex-specific LBD, which was cloned from the GR. When expressed, the protein is localized to the cytoplasm but is targeted to the nucleus upon ligand induction (7, 8, 10). This placed MDM2's access to p53, and subsequent ubiquitination and degradation, also under control of external ligand. To our knowledge, this is the first time that a protein's targeting to the ubiquitin-

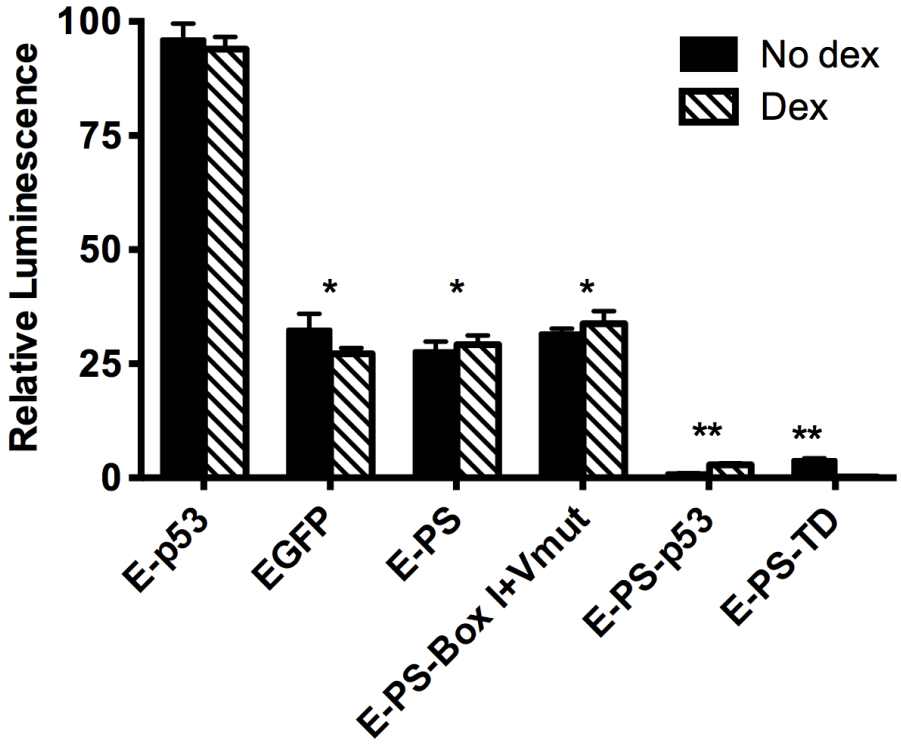


Figure 5.5. Transcriptional activation of PS-p53 constructs. Relative luminescence represents the transcriptional activity of p53 in MCF-7 cells. E-p53, the positive control, showed high transcriptional activity regardless of drug addition. EGFP, E-PS, and E-PS-Box I+V mut did not appear to alter transcriptional activity, either positively or negatively. E-PS-p53 exhibited reduction in transactivation similar to E-PS-TD. Comparisons were analyzed using two-way ANOVA with Tukey's multiple comparisons post-hoc test (* significantly different from E-p53, $p<0.05$; ** significantly different from positive (E-p53) and negative (EGFP) controls, $p<0.05$). Error bars represent standard deviations from three independent experiments (n=3).

proteasome pathway has been controlled by regulating access to subcellular compartments. Knowledge of the ubiquitin-proteasome system is pivotal for understanding the degradation of proteins via the proteasome (49). A protein that is destined for degradation goes through a post-translational modification called ubiquitinylation, which is a process that covalently modifies proteins with ubiquitin via the enzymatic activity of an E1, an E2, and an E3 protein. Ubiquitin is first activated and transferred to the E1 active cysteine residue via a thioester linkage in a process requiring ATP. Ubiquitin is then transferred to the active cysteine residue of an ubiquitin-conjugating enzyme, E2, via a trans(thio)esterification reaction. Ubiquitin ligase, E3, interacts with both E2 and the targeted protein. In general, E3 is important for mediating substrate specificity (50). Subsequently, ubiquitin is ligated to the ε-amino group of a lysine of the target protein through a more stable isopeptide linkage. Since ubiquitin itself contains seven lysine residues, it allows the synthesis of polyubiquitin chains through isopeptide linkages. The process is then repeated giving rise to a polyubiquitin chain, which is recognized by the 19S regulatory caps of the proteasome. In the case of p53 degradation, MDM2 acts as the ubiquitin ligase (E3) while Ubc5 is the ubiquitin conjugating enzyme (E2) (20). Degradation of p53 is dependent on the nuclear exclusion of p53 by MDM2 (51). However, some nuclear proteasomal degradation of p53 can be induced by MDM2 (52).

The mechanism by which our proteasomal protein switch occurs is illustrated in Figure 5.6. We propose that when p53 translocates to the nucleus, it binds to MDM2 and is subsequently ubiquitinated, which results in cytoplasmic and nuclear punctate staining, possibly as ubiquitinated aggregates committed to proteasomal degradation. We confirmed that, indeed, PS-p53 did first travel to the nucleus before

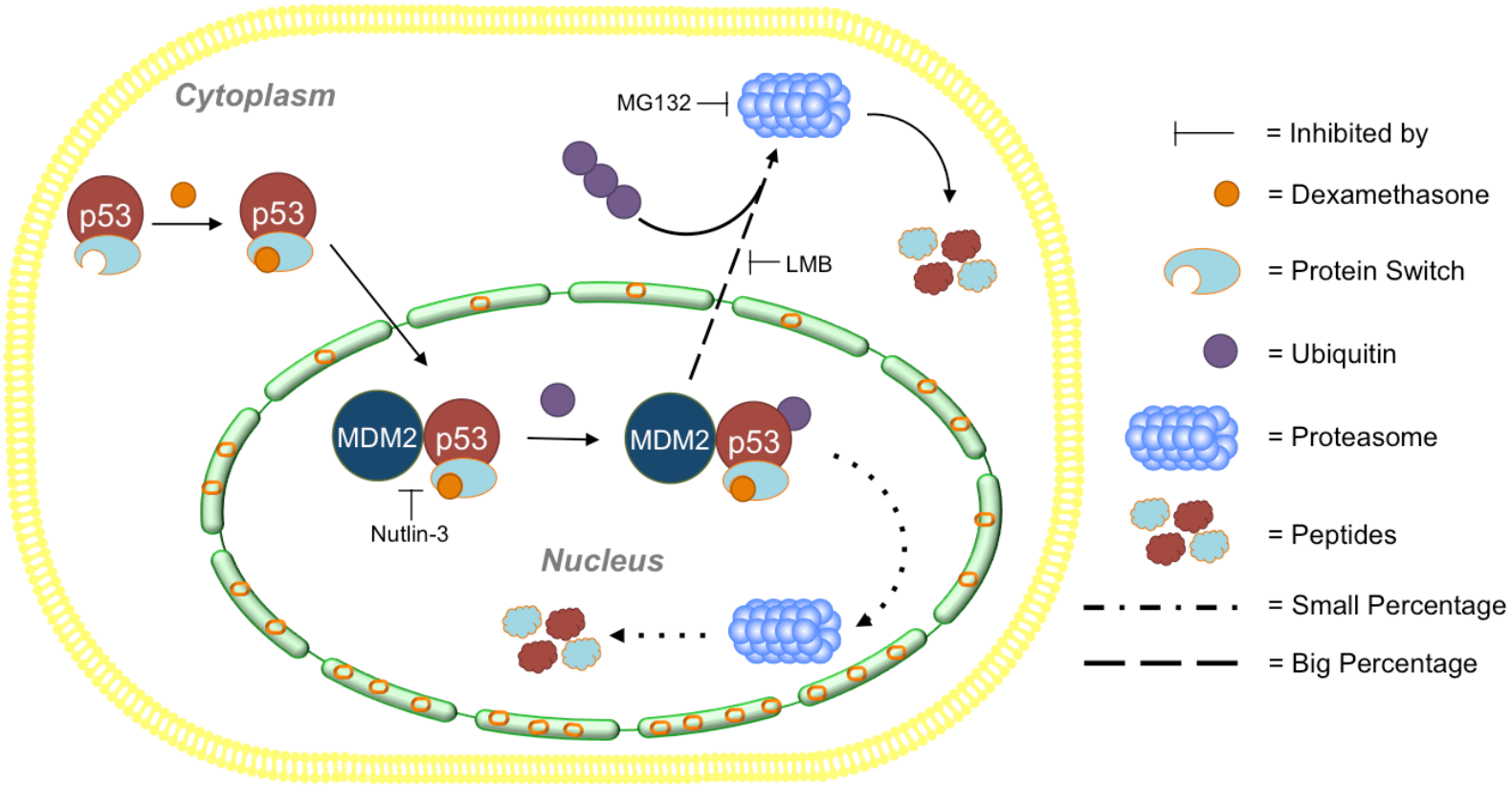


Figure 5.6. The proposed mechanism of proteasomal targeting of E-PS-p53. E-PS-p53 is expressed in the cytoplasm and is targeted to the nucleus upon ligand (dexamethasone) induction. MDM2 binds to p53 in the nucleus causing ubiquitination of p53. A small percentage of the ubiquitinated p53 is sent to nuclear proteasome for degradation. The majority of the ubiquitinated p53 is exported back to the cytoplasm and targeted to the cytoplasmic proteasome for degradation. LMB, nutlin-3, and MG132 can inhibit nuclear export,

being exported to the cytoplasm by inhibiting nuclear export, which demonstrated nuclear accumulation of our construct (Figure 5.2A). When the interaction of MDM2 with p53 was inhibited by nutlin-3, wholesale proteasome-pathway targeted was blocked (Figure 5.2B), verifying that MDM2 was involved in the ubiquitination process. Interestingly, in addition to preventing punctate distribution of our construct, blocking the proteolytic activity of the proteasome promoted nuclear localization (Figure 5.2C), possibly due to lack of ubiquitin availability. Interestingly, fusing the PS to p53 increased its availability to bind to MDM2 compared to E-p53, which shows minimum punctate formation and no degradation compared to E-PS-p53.

There are six lysine residues in p53 that get ubiquitinated by MDM2: K370, K372, K373, K381, K382, and K386 (53). The ubiquitination of p53 by MDM2 begins with the binding of MDM2 to the N-terminal Box-I region of p53. This single interaction is not enough for MDM2 to ubiquitinate p53; small molecule mimetics of this interaction do not prevent the E3 ligase activity of MDM2 (19, 54-57). A second region within p53, called Box-V, was determined to be capable of providing a ubiquitination signal and docking site for MDM2 (54, 58). This lead Wallace et al. proposed a model where binding of MDM2 to the N-terminal Box-I region of p53 allosterically promotes binding of the acidic domain of MDM2 to the Box-V region (58, 59). In other words, the allosteric activation of MDM2 by the N-terminal Box-I region allows a conformation in MDM2 to bind the Box-V region within p53's DBD to allow E2 transfer of ubiquitins to the C-termius of p53.

Indeed, we found that isolating the regions of MDM2 binding (Box I+V) could target the proteasome pathway if a specific destabilizing F270A mutation was made (Figure 5.3A). This phenylalanine residue can block ubiquitination activity by

MDM2, and its removal is associated with hyperubiquitination of p53 (46) The unmutated Box I+V construct lacks the tetramerization domain, which has previously been recognized as necessary for efficient polyubiquitination by MDM2 (46) The F270A mutation may overcome that inefficiency, which explains the degradation seen with Box I+Vmut (Figure 5.4). This construct might be advantageous over E-PS-p53 to future applications for several reasons. Smaller plasmids are easier to transfect, and will leave room for the fusion of other downstream domains. Also, it did not appear to block gene transactivation like full-length p53 fused to the protein switch (Figure 5.5), which might bind to and hence deactivate endogenous p53 because of its intact TD. This was confirmed with similar results when E-PS-TD was used (Figure 5.5). Other truncations of p53 also demonstrated visual proteasome pathway targeting, but these failed to demonstrate degradation. The reason for the difference in fate could be the interplay of monoubiquitination and/or polyubiquitination by MDM2 based on construct localization and dimerization potential (21, 60).

The protein switch was designed to regulate cellular proteins by changing their location, and hence, their function (61, 62). The protein switch has the advantage of being regulated by externally added ligand (1, 7, 8). The proteasomal protein switch can be engineered with a dimerization domain of a protein of interest, which will allow it to “capture” a cytoplasmic endogenous protein and send it for proteasomal degradation upon ligand induction. Since the ubiquitin ligase (E3) is what mediates substrate specificity (50), we envision the use of the constructed proteasomal protein switch as a therapeutic to target oncogenic proteins. Depleting cytoplasmic oncogenic proteins such as Bcr-Abl, survivin, p27, and Raf-1 (4, 63-65) would be a specific treatment for cancer cells. This might prove useful in cancer with

elevated levels of MDM2 such as breast cancer, melanoma, esophageal cancer, leukemia, sarcoma, non-small cell lung cancer, and non-Hodgkin's lymphoma (27). In addition, the EGFP-PS-p53 construct could also be used as a p53-MDM2 binding assay using fluorescence microscopy to screen for inhibitors that could disrupt the p53-MDM2 interaction. Nutlin-3, a MDM2 inhibitor, was used to confirm the feasibility of the assay.

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CHAPTER 6

THE PROTEASOME-TARGETED PROTEIN: DEGRADATION OF THE ONCOGENIC PROTEIN SURVIVIN AND ITS POTENTIAL IMPLICATIONS IN BREAST CANCER

Abstract

Survivin is an inhibitor of apoptosis protein, whose over-expression has been broadly implicated in cancer. Survivin lacks a "druggable" site so conventional means of inhibition are not thought to be possible; agents against survivin currently in clinical trials target the promoter region of the survivin gene, as well as antisense molecules to prevent survivin mRNA translation. In this work, we aimed to disrupt survivin's inhibition of apoptosis by sending it to the ubiquitin-proteasome pathway where it would be degraded by the proteasome. Our proposed proteasome-targeting (protarg) proteins are comprised of an ubiquitin-interacting domain, a ligand-controllable domain, and a dimerization/binding domain that will capture the target protein, i.e. survivin. We tested our proposed full-length estrogen receptor protarg that responds to fulvestrant treatment. Survivin forms a homodimer, so we incorporated the survivin gene into the protarg as a means of capture. To assess the binding capabilities of a survivin homodimer for use in a subcellular trafficking application, we applied the nuclear translocation assay developed in our lab. We also directly assessed the colocalization of survivin with the protarg after induction with

ligand. We concluded that the survivin:survivin homodimer was not robust enough for use as a capture domain for this application.

Introduction

Survivin is a member of the inhibitor of apoptosis (IAP) family of proteins that has been found to be undetectable in differentiated tissue, but overexpressed in embryonic and cancerous cells (1). Survivin is among only a few nodal proteins involved in multiple signaling pathways implicated in tumor progression, and knocking out its function may halt tumor growth (2). Its primary function in healthy dividing cells is to aid in stabilization of the mitotic spindle to aid in chromatid separation and cytokinesis, and to inhibit apoptosis of cells under the stress of cell division. Survivin does this in conjunction with the other members of the chromosome passenger complex (CPC) (3-7). A separate function of survivin that is important for tumor cell survival is its ability to interact with cytosolic proteins, specifically those involved in mitochondrial-associated cell death (Figure 6.1). Through these interactions, caspase-9 is inhibited, which prevents the initiation of mitochondrial-induced apoptosis (8, 9). However, SMAC/DIABLO (second mitochondrial-derived activator of caspase) is released from the mitochondria in response to apoptotic stimuli and can block the inhibition of apoptosis by survivin (10-12).

The expression of survivin increases in cancer cells, possibly as a result of loss of p53 function. Part of p53's normal function is to suppress the *BIRC5* promoter region, which is the regulatory element for survivin transcription. When p53 is lost, as is common in about half of cancers, survivin levels increase, ultimately leading to

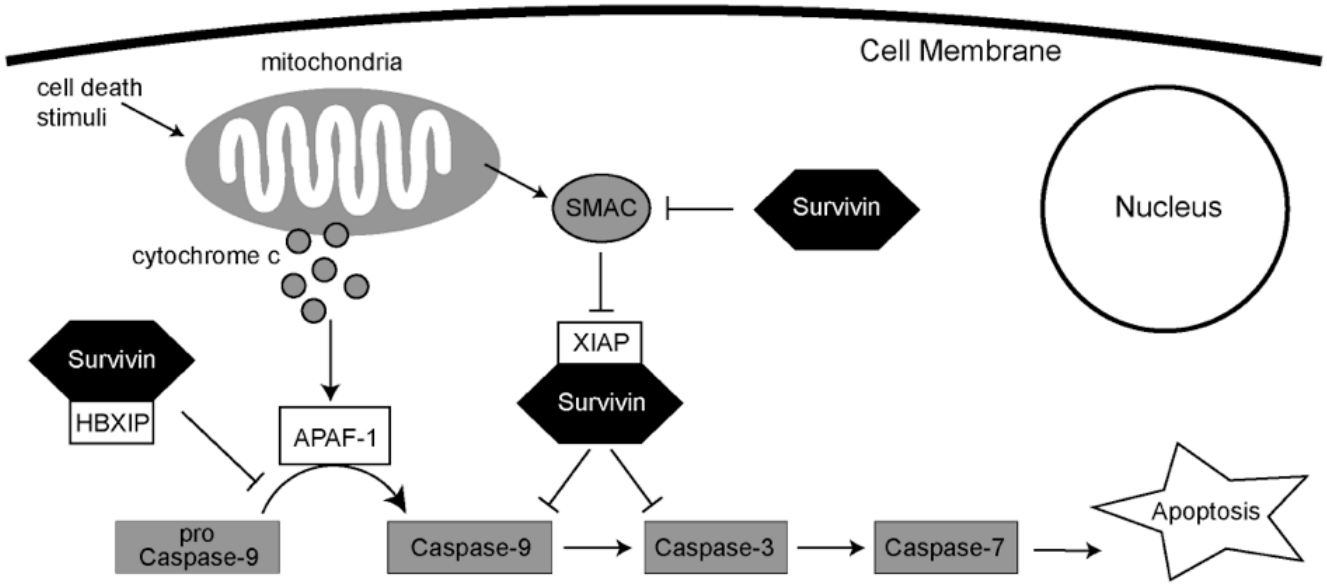

Figure 6.1. Survivin's role in the apoptotic pathway. Cell death stimuli trigger the release of cytochrome c and SMAC from mitochondria, which initiates the apoptotic cascade. Survivin and its binding partners are able to antagonize the pathway by inhibiting SMAC, pro-caspase-9, and caspases 9 and 3.

improved cell survival. (1, 2, 13, 14). This cancer-specific increase of survivin presents itself as an ideal model target for a proteasome-targeted protein system.

In the last chapter, the controlled proteasomal degradation of p53 was described, but here we present a different protarg: one based on full-length estrogen receptor (ER), whose degradation can be controlled by the addition of fulvestrant. We hypothesized that endogenous survivin will be degraded if it binds to the protarg (Figure 6.2).

The human estrogen receptor responds to the endogenous hormone, which causes a conformational change of helix 12 (within the LBD) that allows for coactivator binding and subsequent interaction with EREs. However, the pure antagonist fulvestrant has been found to block all ER transactivation, and to potently induce the proteasomal degradation of the receptor (15-20). We propose to exploit this efficient degradation process to create a ligand-controllable protarg responsive to the ligand fulvestrant. A system that exploits this ligand-controlled degradation could result in an on/off proteasomal switch: on in the presence of fulvestrant, off in its absence. In this study, this ER proteasome-targeted protein was constructed with full-length ER, but mutated to a transcriptionally inactive form (S167A; ER S167A; ER(mut)) (20-22).

Survivin exists as a homodimer (23, 24), and our protargs attempted to utilize this dimeric arrangement for protarg/survivin binding by simply fusing full-length survivin to ERmut. To prevent any oncogenic function imparted by this survivin domain, we introduced a T34A mutation that results in a dominant negative survivin phenotype (7, 25, 26). Figure 6.3 shows the linear arrangement of survivin (A), and now we propose to use the dimerization interface of survivin in our protarg (B); dimerization potential was the main objective of these experiments.

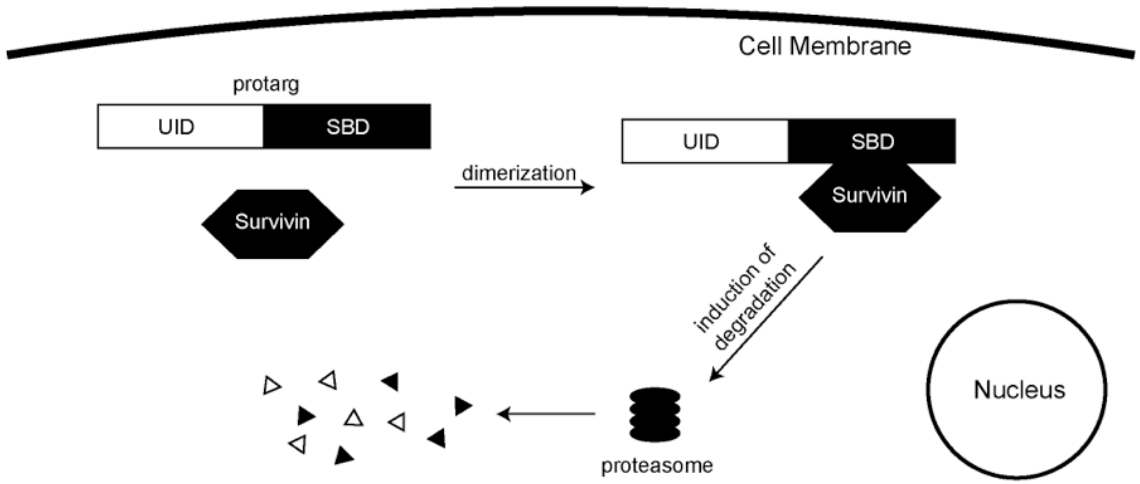

Figure 6.2. Illustration of protarg mechanism. The protarg is composed of an ubiquitin interacting domain (UID) and a binding domain that will target the protein of interest. For this grant, the protein of interest is survivin. The protarg and survivin will dimerize, and upon induction of degradation via the UID, the entire complex will be sent to the proteasomal degradation pathway. The result is loss of survivin protein and function. SBD = survivin binding domain

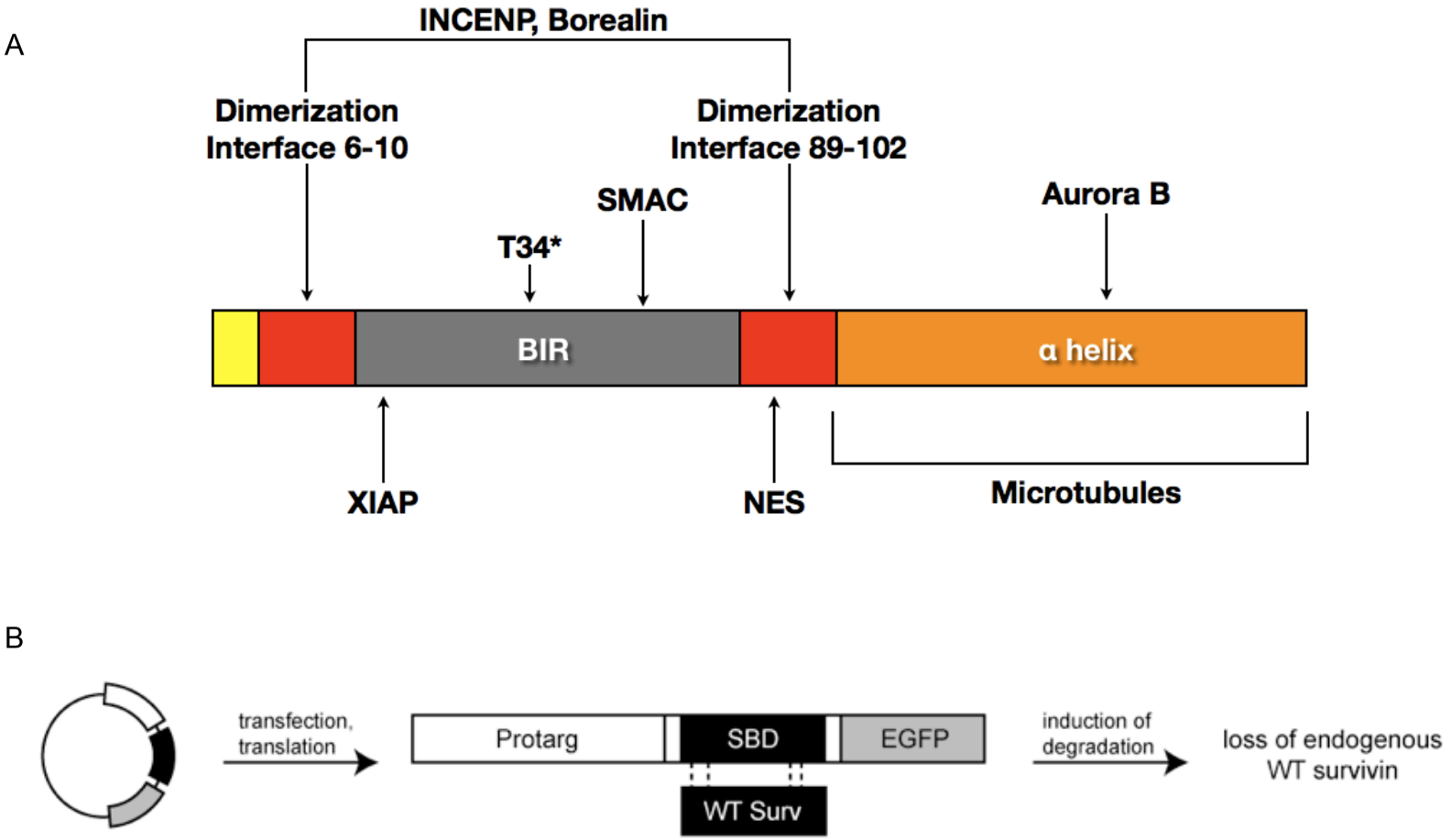

Figure 6.3. Survivin's utility in the protarg system. A. The linear structure of survivin showing binding points and dimerization interfaces. T34* denotes a mutation point that blocks survivin's anti-apoptotic effect. B. Proposed mechanism of protarg binding and degradation of endogenous survivin.

Materials and Methods

Cell Culture

The murine breast adenocarcinoma cell line 1471.1 (a kind gift from G. Hager, NCI, NIH) was grown as a monolayer in DMEM (Invitrogen, Grand Island, NY) supplemented with 10% FBS (Invitrogen), 1% penicillin-streptomycin-L-glutamine (Invitrogen), 0.1% gentamicin (Invitrogen). All cells were maintained in 5% CO_2 incubator at 37ºC.

Plasmid Construction

Survivin (a kind gift from Dario Altieri, The Wistar Institute, Philadelphia) was cloned into DsRed2-N1 (Clontech) by polymerase chain reaction with the introduction of Kpn1 and BamH1 restriction enzyme sites, with forward primer 5'-ATTTAAGGTACCTATGGGTGCCCCGACGTTG-3' and reverse primer 5'-ATTATAGGATCCTTATCCATGGCAGCCAGCTGC-3'.

To clone the protarg plasmids containing the estrogen receptor and survivin, ER was first cloned into the multiple-cloning site of EGFP-N1 plasmid. PCR was used to introduce SacI and EcoRI restriction sites to the 5' and 3' termini, respectively. The primers used were 5'-TAATATGAGCTCATGACCATG-ACCCTCCACAC-3' and 5'-TATAATGAATTCTGACTGTGGCAGGGAAACCCTC-3'. Survivin was then cloned into the resulting ER-EGFP plasmid between the KpnI and BamHI restriction enzyme sites. PCR was used to add these restriction sites to the survivin CDNA with the same primers used in survivin-DsRed2 cloning. To get ER-survivin into the protein switch plasmid, XhoI and ApaI restriction enzyme sites were added through PCR, with forward primer 5'-AATTATCCTCGAG-TTATGACCATGACCCTCCACAC-3' and reverse primer 5'-TAAATTTGGGCCC-

TTATCCATGGCAGCCAGCTGCTC-3'. This fragment was then subcloned into EGFP-PS, resulting in EGFP-PS-ER-survivin. Further mutations were made as follows: To make ERS167A, forward primer 5'-GGCAGAGAAAGAT-TGGCCGCTACCAATGACAAGGGAAG-3' and reverse primer 5'-CTTCCCTTGTG-CATTGGTAGCGGCCAATCTTTCTCTGCC-3' were used with the QuickChange II Site-Directed Mutagenesis Kit (Stratagene/Agilent Technologies, Santa Clara, CA). To create the T34A mutant form of survivin, the QuickChange II Site-Directed Mutagenesis kit was used, with forward primer 5'-GCTGCGCCTGCGCCCCGGAG-CGG-3' and reverse primer 5'-CCGCTCCGGGGCGCAGGCGCAGC-3'. To generate Survivin NES mutant, forward primer 5'-TTCCTTTCTGTCAAGAAGCAGTT-TGAAGAAGCAACCGCTGGTGAATTTTTGAAACTGGACAGAGAAAGAGCC-3' and reverse primer 5'-GGCTCTTTCTCTGTCCAGTTTCAAAAATTCACCAGCGGTTGCT-TCTTCAAACTGCTTCTTGACAGAAAGGAA-3'.

Transient Transfections

Transient transfections were done as previously described (27). Briefly, cells were plated on a clear cover glass in six well plates or live cell chambers with media the day before transfection. Transfections were performed with Lipofectamine 2000 (Invitrogen) per manufacturer's recommendations.

Microscopy and Image Analysis

Cells were imaged as previously described (27-29). Approximately 16 to 24 hours after transfection, cells were viewed on an Olympus IX701F inverted fluorescence microscope. To quantitate protein location, the fluorescent images obtained were analyzed using analySIS® software (Soft Imaging System, Lakewood,

CO) as previously described (28). To obtain average cytoplasmic intensity data for all constructs, 10 representative cells from each experiment were analyzed and averaged from 3 separate experiments (n=3), and are expressed as percent cytoplasmic intensity. Statistical differences between the constructs were determined using one-way ANOVA with Tukey's post-hoc test.

Results

Determination of Co-Localization and Co-Degradation of Survivin-Targeted Protarg

The protarg consists of a protein capable of controlled proteasomal degradation, a target protein binding domain, and EGFP for easy subcellular tracking. The first protarg for testing is based upon the response of the estrogen receptor (ER) to the ligand fulvestrant. The estrogen receptor resides in the nucleus in the un-liganded state, but accumulates in the cytoplasm in a clustered distribution when bound to fulvestrant. This is accompanied by proteasomal degradation in breast cancer cells (17, 18). The estrogen receptor can be mutated at the serine in position 167 to alanine to prevent transcriptional activation, but preserving the degradation potential (20, 30). With mutated ER (ER(mut)) serving as the proteasome-targeted portion, survivin was added to act as a bait domain. Survivin is a homodimer in solution, in an inverted bowtie orientation (23). Survivin can also be mutated to prevent its anti-apoptotic effect by changing amino acid threonine to alanine in position 34 (SurvT34A). This residue does not lie within the dimerization domains, so it should not affect the ability to survivin to dimerize with itself. Microscopic evaluation of colocalization was done to determine if a co-transfected survivin protein would colocalize with the protarg in the pre-degradation

cluster pattern. Figure 6.4 shows the results of this fluorescence microscopy experiment. In figure 6.4 (A), the ER(mut) protarg resides within the nucleus without drug, but when bound to fulvestrant (ICI), the protein does translocate to the expected cytoplasmic clusters within 1 hour. Figure 6.4 (B) shows the results of co-transfecting the ER(mut) protarg with wild-type survivin fused to DsRed, a red fluorescent protein. Without drug, the protarg resides in the nucleus, as expected. However, Survivin-DsRed resides in the cytoplasm. When drug is added, the localization of the protarg changes to cytoplasmic clusters, but the Survivin-DsRed protein does not follow in the clustered distribution. To test there is an interaction occurring with the protarg survivin domain and survivin fused to DsRed, further protein interaction studies were performed, discussed in the next section.

Determination of Interaction by the Nuclear Translocation Assay

As no colocalization was demonstrated in the initial microscopic analysis, we wanted to see if using survivin could form a robust homodimer that could be used to drag a protein within the cell. To specifically test the ability of survivin to bind to itself and be robust enough to allow for intracellular trafficking, the Nuclear Translocation Assay developed in our labwas utilized (29). Briefly, this assay consists of dual transfection of cells with an EGFP based "protein switch (PS)," which translocates from the cytoplasm to the nucleus upon binding with ligand dexamethasone that contains the binding domain for the target protein, and a plasmid encoding the target protein fused to DsRed. The translocation of the EGFP

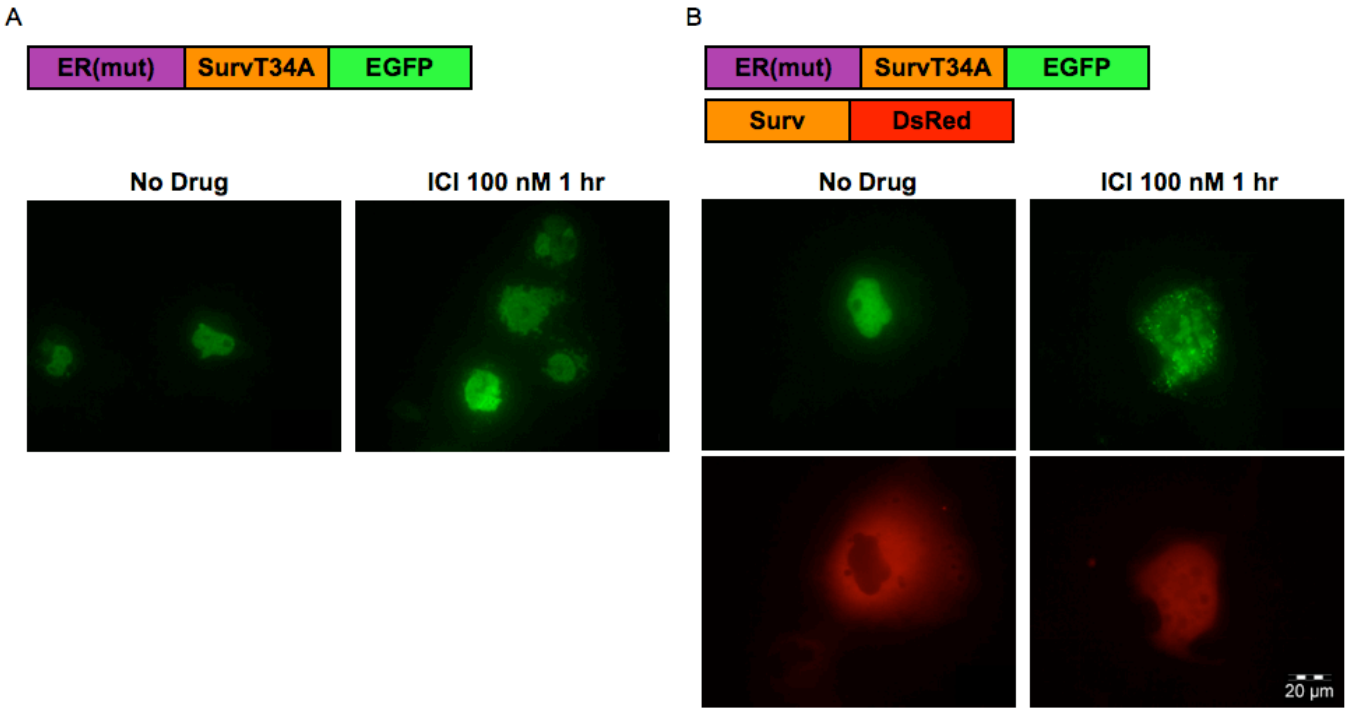

Figure 6.4. Colocalization of ER protarg and wild-type survivin. A. The mutated form of ER (ER(mut)) was fused to mutated survivin (SurvT34A) and treated with ICI 100 nM for 1 hour. The construct localized in cytoplasmic pre-degradation clusters typical to ER antagonism by ICI. B. Cotransfection of the previous plasmid with Survivin fused to a red fluorophore, DsRed2. Drug treatment with ICI caused ER(mut) to localize in cytoplasmic clusters, but no co-localization was seen with with the red and green fluorophores, suggesting that the survivin domains did not dimerize.

protein switch from the cytoplasm to the nucleus should be accompanied by the cytoplasm-to-nucleus translocation of the DsRed plasmid if binding of the target protein is occurring. If no binding occurs, then only the protein switch will translocate to the nucleus. Figure 6.5 (A) shows the results of the negative control constructs: EGFP-PS and Survivin-DsRed transfected independently in 1471.1 cells, and then treated with or without dexamethasone (Dex). When the PS is treated with Dex, it translocates to from the cytoplasm to the nucleus. When Survivin-DsRed is treated with Dex, no change in localization is seen. The results of the fluorescence intensity analysis of these images (Figure 6.5 (B)) reveals a 21.7 ± 3.2 % (mean ± SD) relative nuclear increase for EGFP-PS, and 6.1 ± 6.4 %. These data were significantly different ($p < 0.05$) by an unpaired *t*-test.

When the full ER(mut) protarg was cloned into the PS (EGFP-PS-ER(mut)-SurvT34A) and co-transfected with Survivin-DsRed in COS-7 and 1471.1 cells, the protarg robustly translocated from the cytoplasm to the nucleus as expected (Figure 6.6 (A)). If an interaction was occurring with the mutated survivin in the protarg and wild-type Survivin-DsRed, there should have been a commensurate change in subcellular localization of DsRed. However, there was no appreciable increase in nuclear intensity in the red channel (Figure 6.6 (B)). The nuclear increase of the protarg in COS-7 cells was 30.7 ± 5.1 %, whereas the nuclear increase of Survivin-DsRed was 1.6 ± 5.1 %, which was significantly different by an unpaired *t*-test (p value < 0.05). The results were similar for 1471.1 cells (data not shown).

We questioned the affect that the inherent nuclear export signal (NES) in wild-type survivin has on localization by creating a nuclear export pressure that

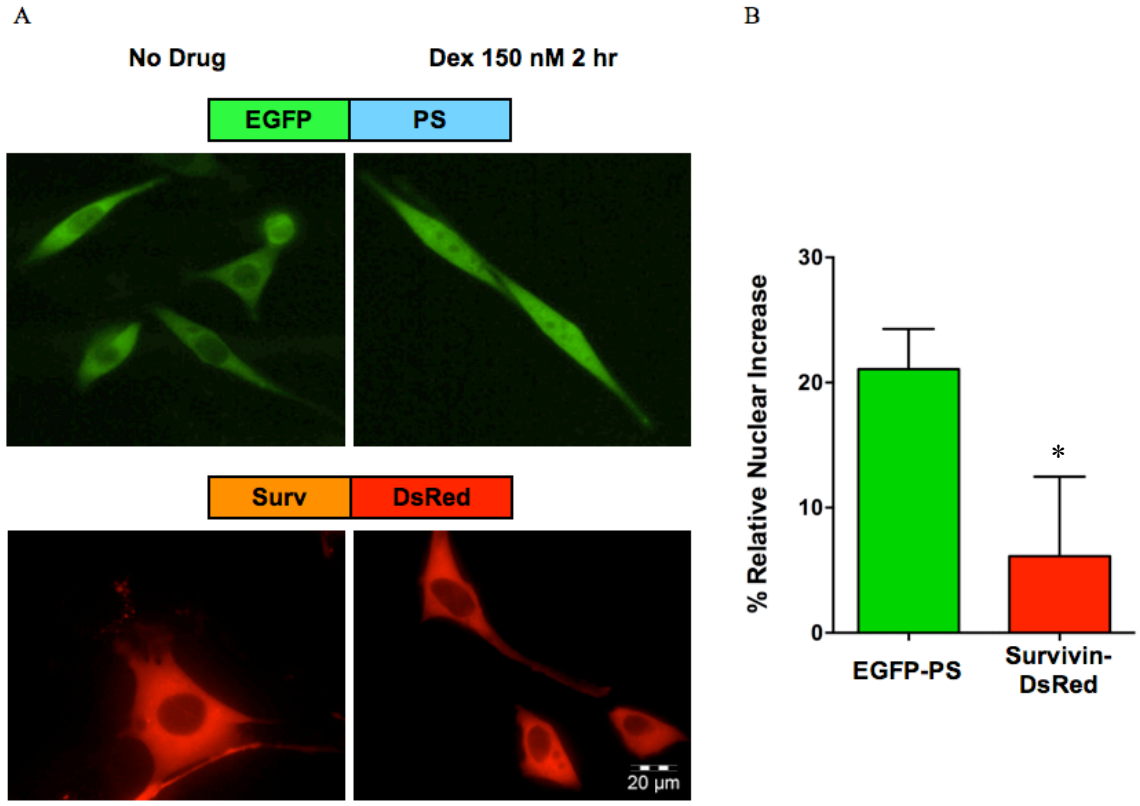

Figure 6.5. Protein switch:survivin colocalization. A. Separate transfection of negative control constructs EGFP-PS (protein switch) and Survivin-DsRed2 in COS-7 cells. The protein switch translocates to the nucleus as expected. Dexamethasone treatment did not significantly increase survivin's nuclear translocation, as expected. B. Quantitation of nuclear translocation by image analysis, presented as % Relative Nuclear Increase. The protein switch increased by 21.7 ± 3.2 %, which was significantly different from Survivin-DsRed, which only changed by 6.1 % ($p < 0.05$).

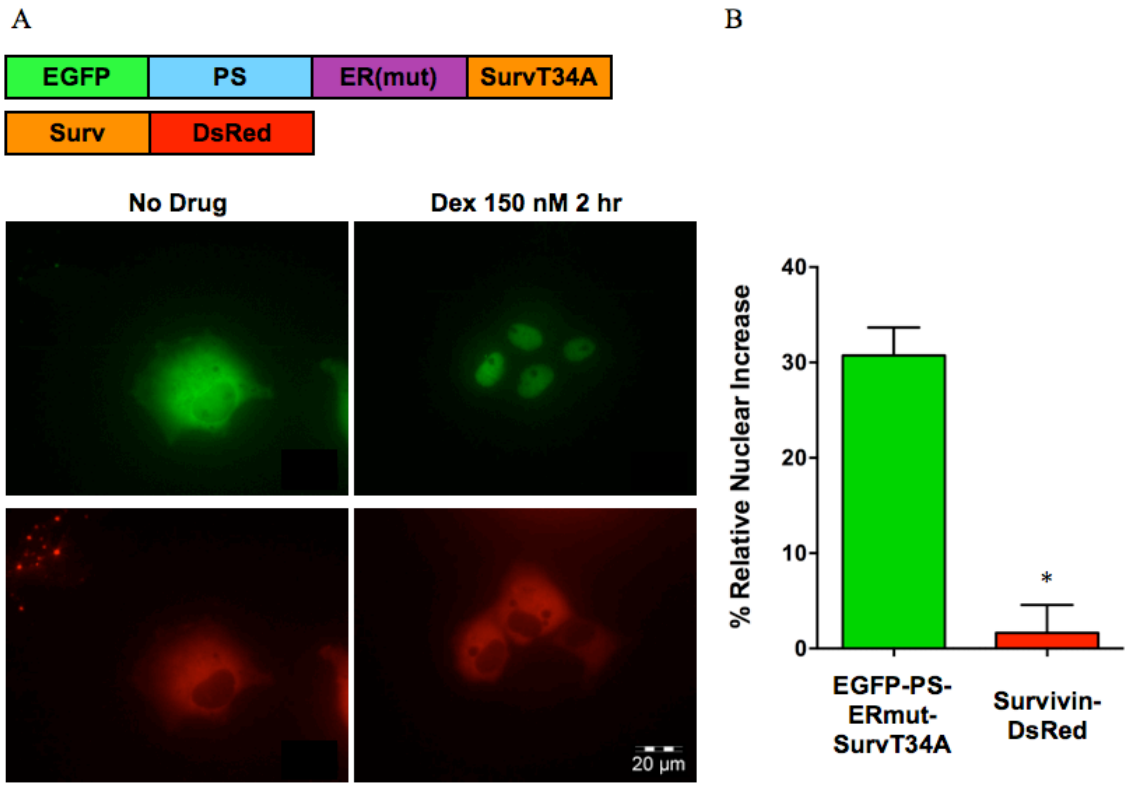

Figure 6.6. Protarg NTA with wild-type survivin. A. Co-transfection of full ER protarg fused to the protein switch construct in COS-7 cells. Survivin-DsRed2. The protein switch translocates to the nucleus as expected. Dexamethasone treatment caused nuclear translocation of the protargs due to the protein switch activity (no degradation because fulvestrant was not present), but survivin-DsRed did not co-translocate and co-localize within the nucleus. B. Quantitation of nuclear translocation by image analysis. The relative nuclear increase (%) for the PS-protarg was 30.7 ± 5.1 %, which was significantly different from Survivin-DsRed, which only changed by 1.6 ± 5.1 % ($p < 0.05$).

cannot be overcome by the homodimer formed with the protarg survivin. To test this question, we removed the NES by site-directed mutagenesis in both the protarg survivin (SurvT34A) and Survivin-DsRed, and repeated the NTA. Figure 6.7 (A) shows the microscopy images. The protarg did again translocate from the cytoplasm to the nucleus, but Survivin-DsRed did not. Image intensity analysis was done, but for only one experiment (analysis of 10 cells, Figure 6.7 (B)).

Lastly, we sought to determine the layout of the protargs was creating a steric hindrance in the dimerization of survivin. The order of the protarg was switched to PS-ER(mut)-SurvT34A-EGFP. The microscopy results of this experiment can be seen in Figure 6.8. Changing the order of the PS-protarg resulted in an increase in nuclear localization before any dexamethasone addition. No overall change was seen in nuclear localization in the PS-protarg or Survivin (Figure 6.8B), and there was no statistical difference between them ($p = 0.4$).

Discussion

Survivin is an inhibitor of apoptosis protein that is overexpressed in most hematopoietic and solid tumors (31). Knocking down its activity has been proposed as a potential treatment option for cancer, but the methods of this are currently in early stages of clinical and translational development, and no proven method of stopping survivin activity exists (32). We hypothesized that using a proteasome-targeted protein that could seek out and bind survivin intracellularly could induce the degradation of survivin and perhaps block its anti-apoptotic effect. The protarg used in this study was based on the full-length estrogen receptor that is targeted to the ubiquitin-proteasome pathway when bound to antagonist fulvestrant through a conformational change in its ligand-binding domain region (18). In order to bind

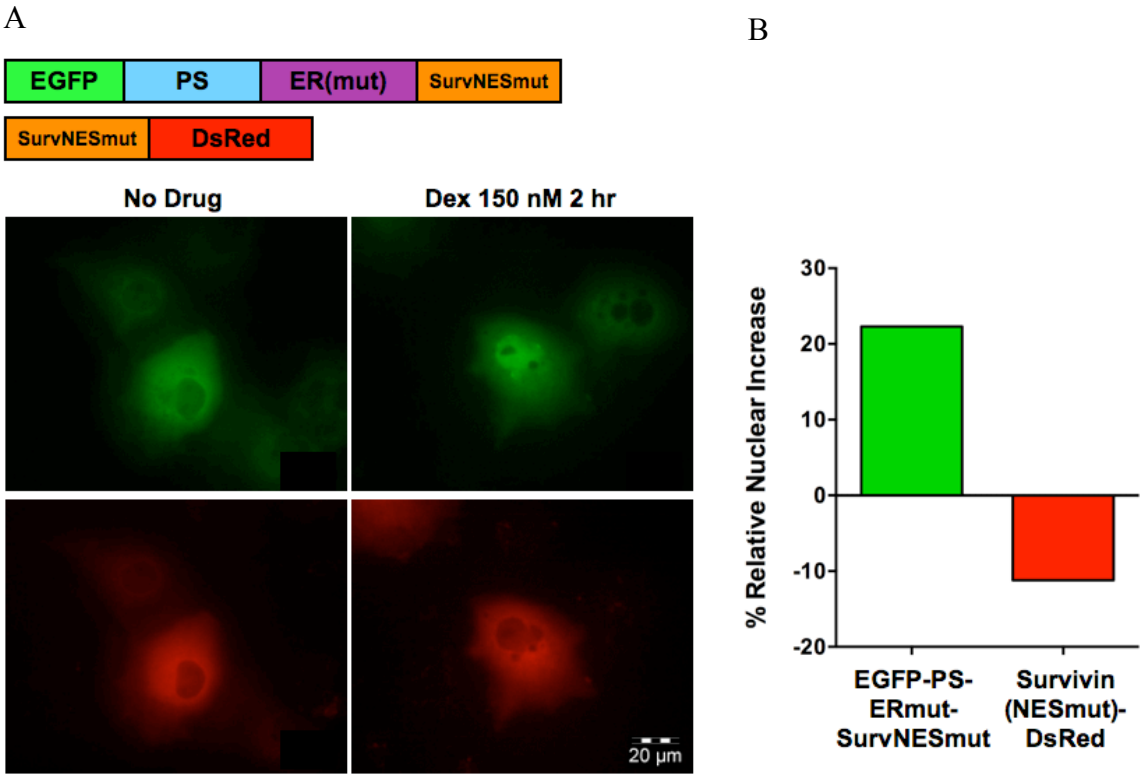

Figure 6.7. NES mutation does not improve colocalization. A. Mutations removing the NES from both survivin domains were made to determine if it would improve nuclear translocation. While the PS-protarg was still able to translocate, the mutations did not improve the translocation of survivin. B. One set of 10 cells analyzed for fluorescence intensity.

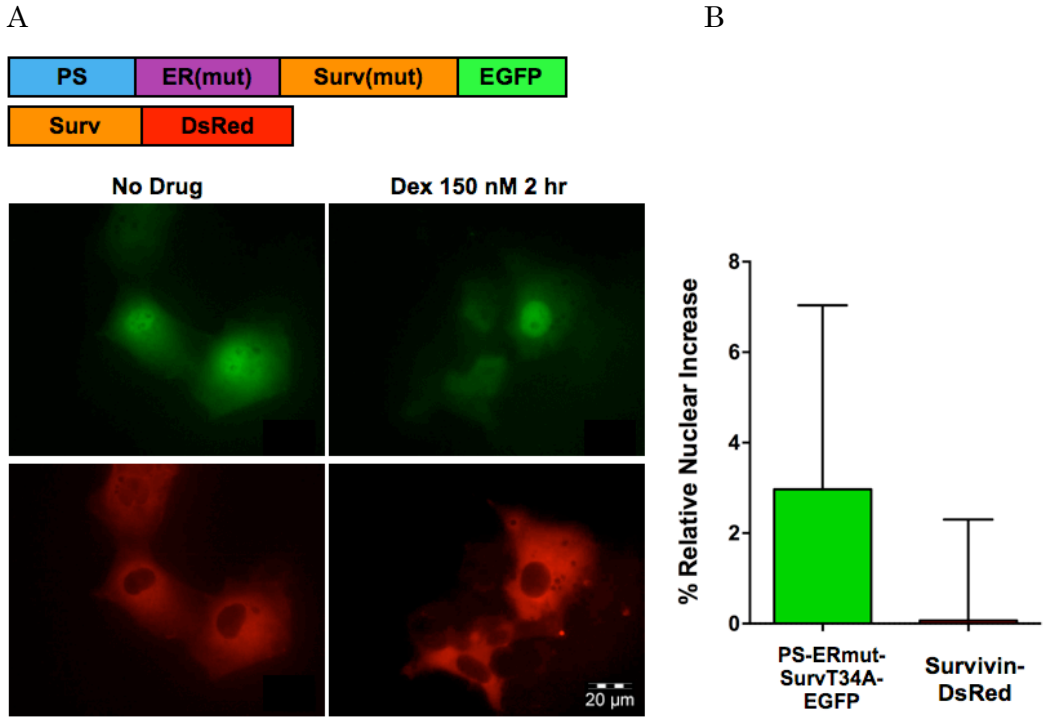

Figure 6.8. Altered orientation does not improve colocalization. A. The linear arrangement of the domains of the PS-protarg was changed to determine of there was a steric affect preventing survivin binding. Removing the EGFP cap on the N-terminus changed the baseline localization of PS-protarg to be more nuclear. No change in survivin localization was seen. B. This change of arrangement resulted in an ability of both constructs to significantly increase their nuclear intensity. There was no difference in relative nuclear increase between these two by unpaired *t*-test. ($p > 0.05$)

intracellular survivin, a survivin binding domain (SBD) was needed. Our first thought was to utilize the native homodimer (24) arrangement of survivin by baiting survivin with itself. Our rationale was that survivin has two dimerization interfaces, and rather than deciphering the exact domain spacing needed to reproduce binding, full-length survivin would suffice. Several mutations were made to ensure that any protein exogenously introduced into a cell would have minimal impact; estrogen receptor was mutated at serine 167 to alanine (20-22) (ER(mut)), and survivin was mutated at tyrosine 34 to alanine which has a dominant negative effect (26).

Before examining actual proteasomal degradation, we tested the ability of survivin to sustain homodimerization through intracellular trafficking. A simple co-transfection of the ER protarg (ER(mut)-SurvT34A), with a survivin-DsRed fusion did not demonstrate any ability of the two constructs to colocalize, either before or after fulvestrant addition. To specifically assess the binding ability of the protarg survivin to a wild-type survivin we used the nuclear translocation assay (NTA) developed in our lab (33). The NTA is an extension of the protein switch that has its subcellular localization under the control of externally administered dexamethasone. A binding domain attached to the protein switch will induce the co-translocation of a target protein from the cytoplasm to nucleus and can be easily visualized by fluorescence microscopy. We found that while the ER(mut) protarg fused to the protein switch (PS-ER(mut)-SurvT34A) could translocate to the nucleus in response to dex, but again, that survivin did not follow, suggesting that there was no binding, or the binding was not durable. Several changes were made to improve the translocation ability, like removing the NES in wild-type survivin, and rearranging the order of the domains in the protein-switch protarg. Neither of these improved nuclear translocation. The most interesting finding of these attempts was that

removing the EGFP fluorophore from the N-terminus of the protein switch changed the nuclear translocation ability completely. The NES and NLS components of the protein switch are in a delicate balance (28), and changing the exposure of the signal sequences tipped the balance in apparent favor of the NLS.

Without proper binding of intracellular survivin, the actual degradation potential could not be assessed. The future experiments in the project must start with seeking a new SBD, perhaps experimenting with the SMAC peptides that have demonstrated IAP binding (34, 35).

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CHAPTER 7

CONCLUSIONS AND FUTURE WORK

Summary

The body of work described in these chapters builds upon the foundation that protein localization control can overcome diseases due to mislocalization or dysfunction. The initial discovery of the "protein switch" concept in the Lim Lab has resulted in a broad array of potential application and localization methods (1-10). Three technologies have been developed with the intent of manipulating protein subcellular localization. There are some elements that are consistent among all three: a ligand-binding domain for inducibility, signal sequences to direct cell compartment specificity, and a domain that interacts with an intracellular target. While none of these technologies are ready yet for any clinical applications, they remain unique among the field of protein targeting, and potentially add to that arsenal of modalities that will aid in researching protein targets for disease therapy, diagnosis, and maybe one day arrive in the clinic with a yet unknown application.

Protein Localization Can Be Controlled with a Protein Switch

The protein switch was initially developed by Charu Kanwal, PhD, in the Lim lab as a "bi-directional on/off switch" to control protein localization between the

cytoplasm and the nucleus (8). With the goal of efficient cytoplasm-to-nucleus translocation in mind, extensive optimization of the nuclear export signal (NES), nuclear localization signal (NLS), and ligand-binding domain (LBD) was undertaken. These NESs and NLSs were tried with combinations of varying signal strengths—as dictated by the binding constant with the respective import/export partner. The result was that these signal sequences needed to be combined to delicately balance the initial cytoplasmic localization against the drive for nuclear localization. A strong NES combined with a weak NLS (MAPKK NES with NLSi, for example) produced a poor increase of protein in the nucleus after ligand induction. Ultimately, a medium-to-strong NLS combined with a medium NES produced the best translocation. Several LBDs were tested with varying NESs and NLSs, including the progesterone receptor (PR), glucocorticoid receptor (GR), estrogen receptor (ER), and ecdysone receptor (EcR). Overall, LBDs combined with the same NES/NLS combination performed similarly, with statistically insignificant differences in translocation measurements. In fact, the NES and NLS combination likely had the greatest influence on translocation ability, and potentially confounded the results of the LBDs tested. Both EcR and ER were tested with the MAPKK export signal. This strong NES may have prevented nuclear import even if the LBD provided the conformational change needed to tip the balance of the dominant signal sequence. The ideal protein switch is one that can be completely controlled by external ligand, so the ideal LBD would not respond to endogenous ligand. The EcR, a steroid hormone receptor found in insects (11-14), is the perfect candidate. Indeed, it is already used in gene-switch systems for this very reason (15). It responds to ecdysteroids that are not present in humans, and humans are constantly exposed to these ecdysteroids without apparent toxicity (15). The finding that MAPKK was a

poor NES choice was determined after the data was analyzed, so a future path for the protein switch is to combined the EcR LBD with the better HIV-SV40/MycA8 signal sequence combination.

Targeting the Nucleus: Future Work

The future work of the protein switch is already underway—meaning that it has already been adapted for use as a protein interaction assay (16), a method for sending oncogenic Bcr-Abl to stop its oncogenic activity (3), and, discussed at length in this thesis, it can be used to control p53's proteasomal degradation (*manuscript submitted for publication*). The nuclear translocation assay (NTA) was developed by Andrew Dixon, PhD, as a means to detect protein interactions by testing colocalization of the proteins of interest in the cytoplasm and nucleus after ligand addition. The "bait" could be fused to the proteins switch, and the protein of interest separately fused to another fluorophore, like DsRed. When co-transfected into a model cell line, like COS-7, the subcellular localization can be monitored by fluorescence microscopy before and after drug addition. If the two proteins interact, a co-translocation to the nucleus after ligand would be expected. If there is not a significant interaction, no co-translocation would be expected. This technology has the potential to be broadly applicable because it could be used as a molecular biology technique without the intent to use it as a therapy. This technology was utilized in the survivin:survivin homodimer study in Chapter 6, with the conclusion that survivin did not form a robust dimer that could withstand cellular trafficking. This may be a limitation of the NTA; interaction sensitivity is traded for easy visualization of the interaction.

Our experience in shuttling proteins from the cytoplasm to the nucleus has yielded valuable lessons that must be learned for future application of this technology. To send a protein from one compartment to another, not only must a robust binding domain be obtained, but also one must undertake a thorough examination of the protein-protein interactions the target protein has that may effect its localization. For example, Bcr-Abl strongly associated with cytoskeletal actin filaments, which increases the complexity of inducing the shuttling of Bcr-Abl. This strong interaction allows Bcr-Abl to "stick" to actin, thereby preventing any induced movement (3). Dixon et al. discovered that using an intracellular antibody against the actin-binding domain of Bcr-Abl blocked the actin interaction, resulting in free Bcr-Abl that can then shuttle between compartments (17-19). A similar approach may be necessary for proteins that do not readily respond to a controlled-localization system.

The research into protein switch applications inspired the other localization technologies presented in this thesis. While optimizing the LBD portion of the protein switch, the response of ER to fulvestrant piqued our interest in investigating proteasomal degradation control, which may be possible in the context of full-length ER bound to fulvestrant. We also discovered that the LBD from ER had a slightly different response to fulvestrant out of context, and this produced the work presented in Chapter 4.

The Estrogen Receptor Ligand-Binding Domain Can Be Utilized to Aggregate Proteins in the Cytoskeletal Fraction

The conformational change of the ligand-binding domain of the ER when bound to fulvestrant increases hydrophobic interactions that contribute to receptor insolubility and aggregation at the cytoskeleton (20, 21). Full-length ER interacts with cytokeratins 8 and 18 (CK8/18) (22, 23), but we found that taking the LBD out of context removed the CK interaction without disrupting insolubility and general cytoskeletal localization. Continuing with the theme of subcellular control, we tested the ability of signal sequences to dictate in which compartment the LBD aggregates. The NES did not significantly change the localization of ER LBD, but adding an NLS did significantly change the baseline localization of the construct. This effect was seen in multiple cell lines, both cancerous (1471.1, MCF-7, HeLa, A2780) and non-cancerous (COS-7), and in CK8/18 positive (MCF-7) and negative (A2780) suggesting that the cytoskeletal target of the LBD alone was different from full-length ER. Confocal microscopy of ER LBD and immunofluorescently labeled CK 18 also corroborated this hypothesis.

Purposefully sending an exogenous protein to the cytoskeleton is a novel concept, and may have a use in investigating protein function. We tested that hypothesis with a mutated coiled-coil (CCmut3) peptide that has activity against Bcr-Abl (24, 25). Bcr-Abl is a well-known protein that is the causative oncogene of chronic myeloid leukemia (CML) (26). This coiled-coil, originally from Bcr-Abl itself, was strategically mutated to bind to Bcr-Abl with higher affinity than a non-mutated form—all while having a lower affinity for itself. In an apoptosis assay in K562 CML cells, a fusion of CCmut3 to ER LBD was able to disrupt Bcr-Abl

signaling and induce apoptosis (2) in the absence of fulvestrant, but the apoptotic effect was lost when fulvestrant was added to the system. Fluorescence microscopy of this construct reveals that indeed the LBD-peptide fusion had aggregated as a result of ligand binding, and this stopped the activity of CCmut3. This both provides evidence of the ability of ER LBD to aggregate a protein and the ability of the CCmut3 designed in our lab to disrupt Bcr-Abl signaling in CML cells. This on/off effect that fulvestrant imparted on CCmut3 could be extended to other rationally designed protein or peptide therapeutics as a means to prove their activity in in vitro studies.

Targeting the Cytoskeleton: Future Work

This initial goal of this project was actually to induce cytoskeletal aggregation of an intracellular protein, namely Bcr-Abl. We again learned that the protein capture and "dragging" is a challenge that is difficult to overcome. The LBD-CCmut3 protein could interact with Bcr-Abl at the site of Bcr-Abl localization, but ligand addition did not induce a localization change of this endogenous protein. The utility of this localization technology may depend on finding robust and specific binding domains for intracellular protein targets like Bcr-Abl, but also other oncogenic proteins like anaplastic lymphoma kinase (ALK) (27), MEK, Raf, and Ras (28, 29). Once a suitable binding domain has been determined, western blot analysis of co-subcellular fractionation, or fluorescence colocalization microscopy, are key to determining if sequestration of the target protein has been achieved. If the target protein can be detected in the insoluble fraction after drug addition, then the effect of sequestering that protein can be tested in downstream assays. However, if no co-fractionation is seen, then that target protein likely has a stronger intracellular

interaction that will need to be disrupted before this system will work. Alternatively, as noted with ERLBD-CCmut3, this system could be used as a molecular biology tool to test the action of therapeutic peptides, or basic protein-protein interactions. Without fulvestrant, the binding domain is able to bind the target protein and the interaction can be assayed for a particular effect (apoptosis, for example). When drug is added, any change in that assay may indicate that the binding domain bound to the target protein was significant.

p53 Can Be Targeted to the Ubiquitin-Proteasome Pathway

Sending proteins to the ubiquitin-proteasome pathway is a concept borne out of the LBD studies in the protein switch optimization. Ligand-induced degradation of ER occurs with fulvestrant, and we expanded upon this mechanism to define a proteasome-targeted protein (protarg) as having a ligand-controllable region, and a region that interacts with ubiquitin and an E3 ligase (ubiquitin-interacting domain; UID). Two such protargs were created and examined in this work: the p53 protarg, and the ER-Survivin protarg.

The interplay of p53 levels and downregulation by MDM2 provided an interesting model system for a protarg. p53 is ubiquitinated by MDM2 via its RING finger E3 ligase activity. Ubiquitination of proteins is a signal for proteasomal degradation, thus p53 is sent to the proteasome via ubiquitination by MDM2 (30). The MDM2/p53 interaction is spatially controlled—both proteins need to be in the same compartment for downregulation to occur (31). This is precisely the type of scenario in which the protein switch becomes especially useful. We hypothesized that p53 fused to the protein switch would localize in the cytoplasm before drug

addition and translocate to the nucleus where it would interact with MDM2, get ubiquitinated, then get sent to the proteasome for degradation. We were correct; p53 did translocate to the nucleus with drug addition, and was subsequently degraded. We found that it must first translocate to the nucleus for any effect to occur, which validates the subcellular compartment control theory. Further, a construct that contains the minimally necessary MDM2 binding regions also performed like full-length p53, but had the added benefit of not modulating p53 gene translocation. The protein-switch p53 construct reduced p53 gene transcription when compared to the negative control, but the truncated version did not. The implication is that this exogenously introduced protein will have fewer negative affects on the cell in which it is introduced.

Targeting the Ubiquitin-Proteasome Pathway: Future Work

The basic mechanistic characterization of the p53 protarg is largely complete, but proving that true proteasomal degradation is occurring is the next major step. The work presented in this dissertation has shown that loss of GFP fluorescence correlated with a change in localization of PS-p53 constructs. While it is tempting to seek to prove that colocalization of the protarg with the proteasome precedes loss of fluorescence, our unpublished data have repeatedly shown that colocalization is not possible to visualize. We speculate that the colocalization of the protarg with the proteasome is too transient; once the protein reaches the proteasome it is immediately degraded. The clusters of fluorescence seen in the images do not depict actual proteasomal localization, but rather that clusters of protein preceding degradation. More experiments are needed showing that the proteasome alone is

responsible for loss of fluorescence. A similar flow cytometry based assay as used in Chapter 5 may be used with a proteasomal inhibitor, along with a protein synthesis inhibitor, to show that without a functioning proteasome there is no loss of fluorescence. The key to this experiment is optimizing the time of drug addition. Concomitant administration of an inhibitor of the proteasome at the same time as dexamethasone may not yield true results without suitable drug addition timing. Western blots may also be a useful as supportive evidence that protein levels are decreasing as a result of dexamethasone administration. A blot of subcellular fractionation will assure that the disappearance of a protein is not simply due to solubility in a specific buffer.

Next, a suitable application of this technology remains to be determined. The same challenges faced by the ER LBD-cytoskeleton protein are faced here: a suitable intracellular protein needs to be identified, and a binding domain located or rationally designed. Further, another drawback of this technology is that it relies upon the cell to produce MDM2 in order for p53 to be ubiquitinated. In cells with low MDM2 levels, this protarg may not function at all. Further investigation into cell specificity of the p53 protarg needs to be done, and a possible result is that this protarg is cancer-specific. Cells with high MDM2 activity, such as those in certain cancers (sarcoma, for example) (32) may quickly and efficiently send the p53 protarg to the proteasome, and hopefully with the target protein to which it has bound.

Survivin Does Not Form a Durable Dimer Interface for Protarg Use

We tested the ability of survivin to dimerize with itself in order act as a capture domain in our protarg construct. Rather than using the p53 protarg, we

attempted to characterize the degradation potential of ER as a UID. When ER binds fulvestrant, it is quickly sequestered with cytokeratins in the nucleus and cytoplasm, and proteasomal degradation shortly follows (21, 23). This, in combination with survivin as bait, served as our anti-survivin protarg. We took into consideration the potential of our construct to actually do harm, i.e. survivin acting as an inhibitor of apoptosis and ER transactivating genes unnecessarily, so we made strategic mutations in each to prevent this occurrence (33-35). Colocalization of our protarg construct with a co-transfected wild-type survivin/fluorophore fusion was not demonstrated in two separate methods. Direct visualization of pre-proteasomal clusters of the ER bound to fulvestrant shows that survivin remained broadly localized within the cell. Using an assay that visibly demonstrates binding of two proteins (16) also showed that wild-type survivin did not bind to nor translocate with the protarg. Even though we did not see any survivin binding activity, it was an opportunity to deploy other technologies developed in our lab that stemmed from controlling protein localization: the nuclear translocation assay. Also, a valuable lesson learned in these experiments was that removing the enhanced green fluorescent protein (EGFP) cap from the N-terminus of the protein switch blocked localization control with dexamethasone. The obvious conclusion is that EGFP was assisting in masking the NLS prior to drug addition, and its removal exposed the NLS to the import machinery causing nuclear accumulation regardless of drug addition.

The future work on survivin must delve into a better binding domain against survivin. One place to start is using SMAC peptides to lure survivin. SMAC (second derived mitochondrial-derived activator of mitosis) is released from the mitochondria in response to apoptotic stimuli. A cleavage of SMAC occurs that exposes an N-

terminal amino acid sequence that binds the Inhibitors of Apoptosis (IAPs), of which survivin is a member (36-42). Short peptides comprised of this N-terminal amino acid sequence have been made, and their binding potency characterized. We could incorporate a coding sequence into our protarg that when expressed by the cell produces an IAP biding region. This region, in association with ER, could produce a viable protarg that could degrade intracellular survivin.

Localization-Controllable Proteins: Future Perspectives

The three modalities of protein localization control presented in this dissertation have a shared feature of ligand inducibility. The appeal of this approach is that the action of induced protein translocation can be specifically timed, with the potential of cell specificity based on ligand choice. However, it may not be necessary to use a complicated ligand-controllable system. The Lim lab is already underway on direct targeting of p53 to the mitochondria (10). The control element has been removed, and all that remains is a mitochondrial targeting signal fused to p53. When this fusion protein is expressed, intracellular processes immediately recognize the targeting signal and the whole protein is sent to the mitochondria. When p53 is sent to this organelle, the intrinsic apoptotic pathway is initiated and results in increased cell death. This is an example of a system that may have greater therapeutic potential due to its more simplistic mechanism. A similar approach may be explored with nuclear-targeted proteins: inclusion of nuclear localization signals; cytoskeletal-targeted proteins: fusing a binding domain to a highly hydrophobic domain; and proteasomal-targeted proteins: inclusion of a degradation signal, such as N-degrons or ubiquitin-fusion proteins (43-45).

The delivery of these therapeutics is another major hurdle that lies ahead. The delivery of the mitochondrially-targeted p53 protein is also underway in the Lim lab with adenoviral vectors. All of the gene delivery work done in this dissertation was done with commercially available lipopolymers that are efficient in vitro, but not feasible in vivo. Adenoviral delivery has become a popular method of gene delivery, and is currently being tested in clinical trials, most notably with Advexin®, the US cognate to the Chinese Gendicine® adenoviral p53 vector (46). The adenovirus is capable of efficient infection of nondividing or differentiated cells (47), and can be engineered with tropism toward a specific tissue type (48). However, the utility of adenoviral vectors is limited by preexisting immunity toward adenovirus, infection of off-target cells, and vector immunogenicity (49). Nevertheless, it remains a promising method of efficient delivery of engineered gene therapy products.

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